SCIENTIFIC PROTOCOLS FOR THE *IN VITRO* AND *IN VIVO* EVALUATION OF ANTIMICROBIAL AND IMMUNOMODULATORY PROPERTIES OF NATURAL COMPOUNDS FOR APPLICATION IN FISH

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Title: Scientific protocols for the *in vitro* and *in vivo* evaluation of antimicrobial and immunomodulatory properties of natural compounds for application in fish

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www.italy-croatia.eu/adriaquanet

Università degli Studi di Udine, Udine.

CONTENTS

PREFACE

The contributions to the present manual were assembled with the goal of providing the reader with a practical guide regarding some of the more common methods that are currently used in fish immunology and pathology for the evaluation of antimicrobial and/or immunomodulatory properties of natural compounds in fish species, with the perspective of their use in aquaculture for the control of infectious diseases as alternative substances to the conventional drugs commonly applied in fish farms.

The manual is intended to describe the basic approach that can be follow for identifying new drug/immunostimulant candidates and defining their mechanism(s) of action, moreover it includes some guidelines for the selection, development and optimization of laboratory and in field assays to be performed for the *in vitro* and *in vivo* screening of:

- antibacterial activity of natural compounds against fish pathogens;
- antiparasitic activity of natural compounds against fish pathogen;
- immunostimulant effects of natural compounds on fish leukocytes;
- possible cytotoxicity of natural compounds on fibroblast-like cell line derived from *Sparus aurata* L. caudal fin by the detection of cell viability;
- efficacy of a candidate natural compound as new therapeutic agent for the treatment of fish bacterial infection after an experimental small-scale challenge trial;
- efficacy of a candidate natural compound as new dietary immunostimulant for the prevention of fish bacterial infection after an experimental small-scale trial.

This manual is dedicated to academic researchers, students, postdoctoral fellows, and laboratory staff, therefore detailed methodological protocols are presented on a level designed to ensure that anyone with a background in biological research will be able to execute and validate the experimental assays but also will be aided to identify and correct eventual technical difficulties in order to overcome a particular issue, so as to facilitate the establishment and implementation of the procedures in appropriately equipped research laboratories and/or experimental facilities. The protocols have been extensively used by the researchers of the AdriAquaNet project and have been prepared from the original methodologies, in some instances with slight modifications as described.

Reagents required for each protocol are itemized in the materials list before the procedure description. Many of them are stock solutions, others are commonly used buffers or media, while others are solutions unique to a particular protocol. Throughout the manual, we have recommended commercial suppliers of chemicals, biological materials, and equipment. In some cases, the noted brand has been found to be of superior quality or it is the only suitable product available in the marketplace. In other cases, the experience of the authors is limited to that brand. In the latter situation, the reader is advised to search for the most suitable materials and tools to use for the experiments.

Some protocols contemplate the use of live animals. Prior to conducting any laboratory procedures with fish, the experimental approach must be submitted to the approval of the Institutional Animal Care and Use Committee and must comply with the appropriate governmental regulations regarding the care and use of laboratory animals. A written authorization from the Animal Care and Use Committee is absolutely required prior to undertaking any live-animal studies. Some specific animal care and handling guidelines are provided in the protocols where fish are used, but the check with governmental guidelines is often necessary to obtain more extensive information.

EXPERIMENTAL APPROACH FOR *IN VITRO* AND *IN VIVO* SCREENING OF ANTIMICROBIAL AND IMMUNOMODULATORY PROPERTIES OF A PANEL OF NATURAL COMPOUNDS

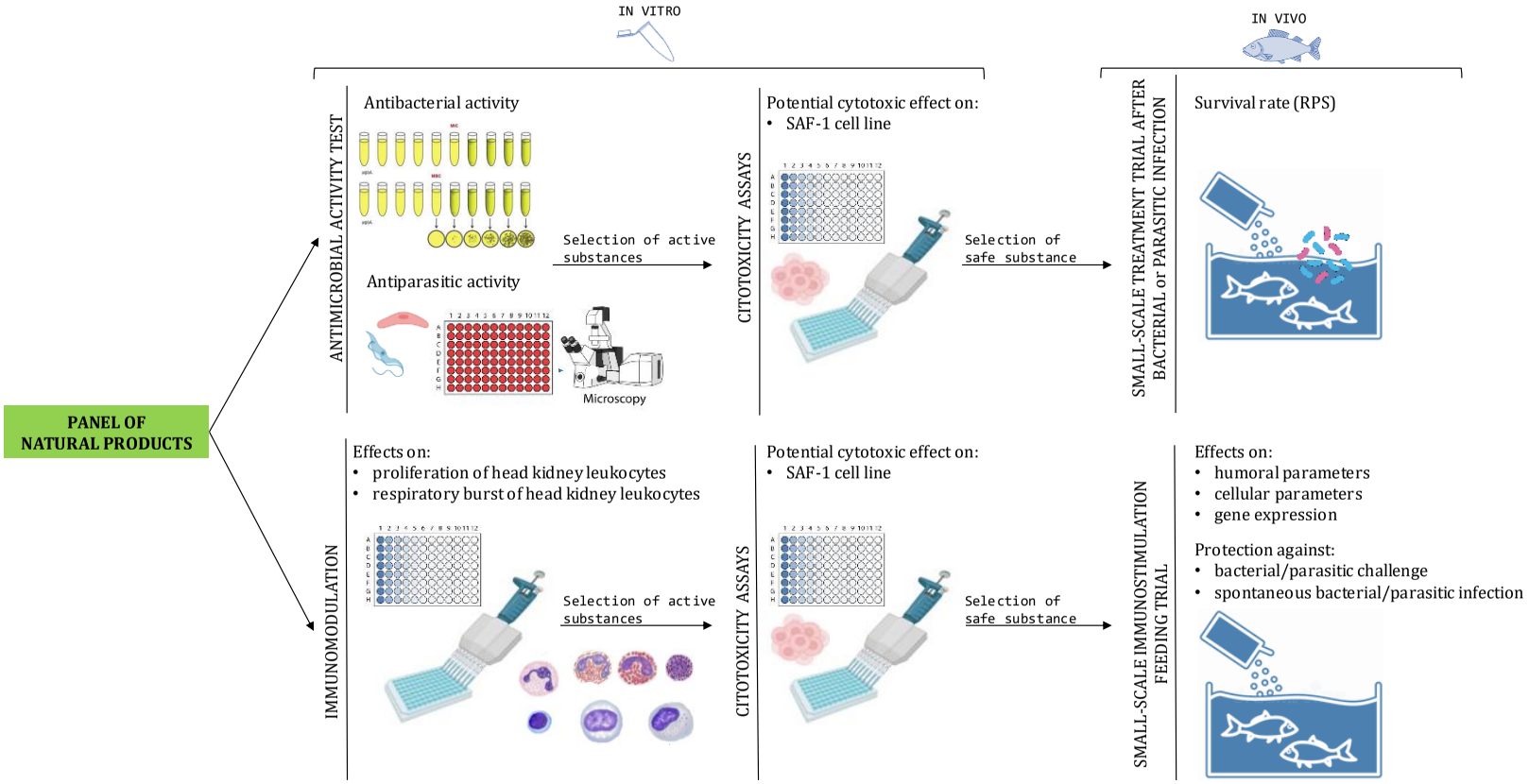

Fig. 1. Protocol for the screening of antimicrobial and immunomodulatory properties of a panel of natural compounds developed within the framework of AdriAquaNet project. The natural compounds can be tested adopting the broth dilution method to determine their antibacterial and antiparasitic activity; moreover they can be tested on fish leukocytes in order to evaluate their immunomodulatory properties (i.e. effects on cell proliferation and respiratory burst activity); subsequently, they can be incubated with a fish cell line (i.e. SAF-1 from Sparus aurata) that is then submitted to a MTT viability assay, in order to evaluate their potential in vitro cytotoxicity. Then, the substance demonstrated positive antimicrobial and/or immunostimulant effects but not showing cytotoxic effects can be selected and addressed to a small-scale in vivo trial, aimed to determine if it is effective in the treatment of infection diseases when added in the diet (medicated feed) or in enhancing fish immune response and preventing infections when used as dietary immunostimulant.

IN VITRO ANTIBACTERIAL ACTIVITY OF A PANEL OF NATURAL COMPOUNDS AGAINST FISH PATHOGENS

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REAGENTS

Dimethyl sulfoxide (DMSO) - Merck Life Science s.r.l (MI, Italy)

Culture broth and agar suitable for the selected bacterial strain (i.e. trypticase soy broth or agar)

Oxytetracycline - Merck Life Science s.r.l (MI, Italy)

Phosphate buffer saline (PBS) pH 7.2

Sodium chloride (NaCl) - Merck Life Science s.r.l (MI, Italy)

Natural compounds (i.e. MNPs, AMPs, probiotic supernatants, medicinal plant extracts or active metabolites)

GROWTH AND PREPARATION OF BACTERIA

- Inoculate the bacterial strain of interest in suitable culture broth (with or without 1,5% NaCl);
- Growth the bacteria overnight at a temperature optimal for the bacterial strain, until the logarithmic phase of growth (Fig. 2);
- Collect the bacteria after centrifugation at 2000*xg* for 20 min;
- Suspend the bacteria in sterile PBS pH 7.2 or physiological solution;

Fig. 2 Bacterial growth in culture broth.

- Standardize the concentration of each strain by spectrophotometrically adjusting the optical density (OD) of the bacterial suspension at wavelength = 610 nm to 1.0 (based on experimental data, this OD value correspond to a concentration of 1×10^9 CFU/ml for some of the common fish bacterial pathogens) by using a microplate reader (i.e. Tecan Sunrise™, MI, Italy);
- Dilute the bacteria in sterile PBS or physiological solution, in order to reach a bacterial concentration of 5×10^5 CFU/ml;

- Spread 50 µl of 10^5 and 10^6 dilutions of bacterial suspension onto solid agar medium in plates (in duplicate);
- Incubate the plates at an optimal temperature for the fish pathogen for 24 h;
- After incubation, count the number of colony forming units (CFU), in order to confirm the bacterial concentration.

BROTH MICRO-DILUTION METHOD FOR MIC AND MBC ASSESSMENT

(Bulfon *et al*. 2014)

MINIMUM INHIBITORY CONCENTRATION (MIC) = lowest concentration (µg/ml) of each compound that completely inhibited the visible growth of pathogen, when compared with the control wells with bacterial growth (Konè *et al*. 2004).

MINIMUM BACTERICIDAL CONCENTRATION (MBC) = lowest concentration (µg/ml) of each compound that is associated with no visible bacterial growth on subcultures (Konè *et al.* 2004).

DAY 1 – MICROPLATE PREPARATION FOR MIC DETECTION

- Prepare two-fold serial dilutions of natural compounds diluted in sterile culture broth (100 µl/well, in triplicate) in U bottom 96-well microplates;
- Add 50 µl/well of bacterial suspension at concentration of 5×10^5 CFU/ml;
- Incubate the microplates for 24 h at a temperature optimal for the bacterial strain.

Be sure to also include in each test:

a. NATURAL COMPOUND STERILITY CONTROL: wells with culture medium and natural compounds without bacteria, in order to exclude the contamination of the tested natural compounds;
b. CULTURE MEDIUM STERILITY CONTROL: wells with culture medium only, in order to exclude the contamination of the culture medium;

c. REFERENCE ANTIBIOTIC: wells with two-fold serial dilutions of a reference antibiotic diluted in sterile culture broth and bacteria, in order to compare its antibacterial activity with that of the natural compounds;

d. BACTERIAL GROWTH CONTROL: wells with bacteria and culture medium, in order to verify their effective growth at the experimental conditions;

e. SOLVENT EFFECT CONTROL: wells with bacteria and two-fold serial dilutions of the solvent used for the preparation of natural compounds (if different from water and therefore potentially toxic to bacteria) diluted in sterile culture broth, in order to exclude its potential inhibitory effects on the fish pathogen.

DAY 2 – MICROPLATE READING, MIC DETERMINATION AND MICROPLATE PREPARATION FOR MBC DETECTION

- Examine the bacterial growth in the microplates by observing the turbidity of the wells, in order to detect the MIC value of each tested natural compound.

Note: the absence of any turbidity or bacteria pellet in the bottom of the wells indicates the absence of bacterial growth whereas the presence of turbidity or bacteria pellet on the bottom of the wells indicates the bacterial growth (Fig. 3).

- Transfer 10 µl of medium from the wells with no visible growth in fresh sterile culture broth (240 µl/well) in another U bottom 96-well microplates (in duplicate);
- Incubate the microplates for 24 h at a temperature optimal for the bacterial strain.

Be sure to also include in each test:

a. CULTURE MEDIUM STERILITY CONTROL: wells with culture medium only, in order to exclude the contamination of the culture medium;
b. BACTERIAL GROWTH CONTROL: wells with bacteria and culture medium, in order to verify their effective growth.

DAY 3 – MICROPLATE READING AND MBC DETERMINATION

- Examine the bacterial growth in the microplates by observing the turbidity of the wells, in order to detect the MBC value of each tested natural compound.

Note: wells with absence of any turbidity or bacterial pellet in the bottom of the wells indicates the absence of bacterial growth and a bactericidal mode of action of the natural compound whereas the presence of turbidity in the wells or bacterial pellet on the bottom indicates the bacterial growth and a bacteriostatic mode of action of the natural compound.

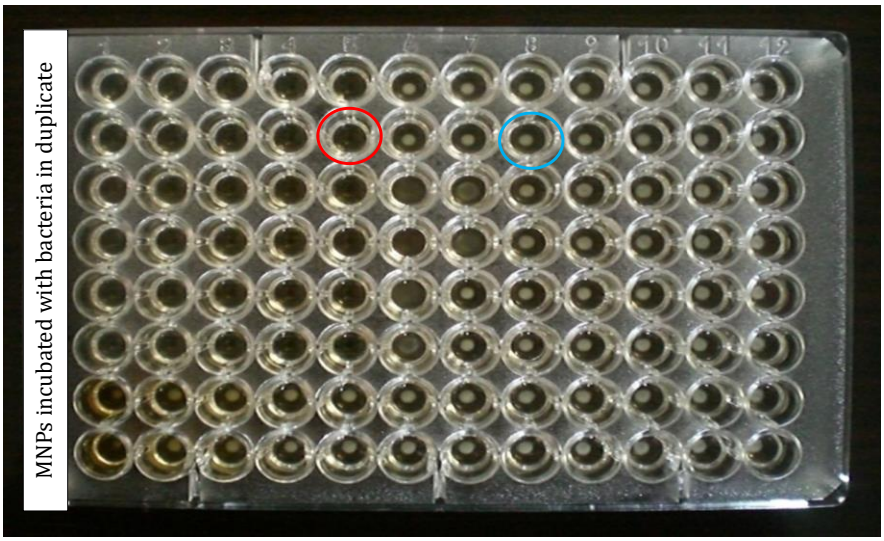

Fig. 3. Example of U-bottom 96-well microplate used for the evaluation of natural compounds antibacterial activity through the broth dilution method. The red circle indicates a well with clear content and without bacterial growth. The blue circle indicates a well with the presence of bacterial pellet on the bottom and therefore the bacterial growth.

IN VITRO ANTIPARASITIC ACTIVITY OF A PANEL OF NATURAL COMPOUNDS AGAINST FISH PATHOGENS

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REAGENTS

Dimethyl sulfoxide (DMSO) (or Ethanol or Methanol) – Sigma-Aldrich

Filtered, autoclaved sea water

Natural compounds (i.e MNPs, AMPs, probiotic supernatants, medicinal plant extracts or active metabolites)

PARASITES

Fish ectoparasites, e.g. *Ceratothoa oestroides*, *Sparicotyle chrisophrii* or any other parasite of interest

It is recommended to use pullus II stage of *C. oestroides* and adult stage of *S. chrisophrii*

- Collect pullus II stages of *C. oeastroides* from gravid female *C. oeastroides*;

- Collect adult stages of *S. chrisophrii* from infected gills of gilthead sea bream (*Sparus aurata*): to avoid the damage to the parasites, fish need to be euthanized, the gill arches have to be excised and the parasites must be carefully removed by a dissecting needle.

ANTIPARASITIC ACTIVITY

NOTE: This protocol can be applied also to endoparasites.

DAY 1 – ANTIPARASITIC ACTIVITY OF SELECTED NATURAL COMPOUNDS (PRELIMINARY EXPERIMENTS)

- Dissolve the natural compounds in an appropriate solvent (DMSO, ethanol or methanol), depending on their chemical form;

- Prepare 10 mM stock solution of each natural compound by diluting it with filtered and autoclaved sea water;

- Prepare four serial dilutions of natural compounds in filtered and autoclaved sea water, starting from the 10 mM stock solution (i.e. 1 mM, 0.1 mM, 0.01 mM, and 1 µM);

- Fill 6-well plates with 2 mL of each dilution of natural compounds, in triplicate;

- Place 10 parasites in each well and check them under a stereomicroscope for visible signs of mechanical damage;

- Incubate the parasites in dark at room temperature for up to 24 hours post exposure;

- Record the number of live/dead parasites after 1, 4, 12 and 24 hours post exposure.

Be sure to also include in each test:

a. NATURAL COMPOUND STERILITY CONTROL: wells with filtered and autoclaved sea water and natural compounds without parasites, in order to exclude the contamination of the tested natural compounds;

b. SEA WATER STERILITY CONTROL: wells with filtered and autoclaved sea water only, in order to exclude the contamination of medium;

c. REFERENCE ANTIPARASITIC SUBSTANCE: wells with appropriate dilutions of a reference antiparasitic substance diluted in in filtered and autoclaved sea water and parasites, in order to compare its antiparasitic activity with that of the natural compounds;

d. PARASITE CONTROL: wells with parasites and filtered and autoclaved sea water, in order to verify their vitality at the experimental conditions;

e. SOLVENT EFFECT CONTROL (negative control): wells containing parasites and the solvent used for the preparation of natural compounds (if different from water and therefore potentially toxic to parasites) to appropriate final concentrations in filtered and autoclaved sea water, in order to exclude its potential inhibitory effects on the fish pathogen.

NOTE1: Parasites are usually considered dead if no type of autonomous movement is observed within 5 s after gentle probing with a fine needle. In the case of *C. oestroides*, no movement of pereopods (limbs) is observed.

NOTE2: If the initial concentration of the natural compounds is low, all of the above test concentrations can be downscaled for one order of magnitude to account for this.

NOTE2: Higher concentrations of serial dilutions of natural compounds can be prepared and added to 2 mL of sea water in each well so that the final concentrations reach the ones mentioned above (e.g. 1.8 mL of sea water and 200 µL of 10 mM natural compound, resulting in a final concentration of 1 mM).

DAY 2 – DOSE-RESPONSE EXPERIMENTS

- Based on the antiparasitic activity of natural compounds detected in the preliminary experiments, tailor a range of at least 5 concentrations to be tested in the dose-response assay;
- Scale down the range of concentrations to an appropriate order of magnitude, so that each natural compound reaches 100% mortality at 4 h post-exposure.

I.e. if 100% mortality at 4 h post-exposure was achieved with 0.1 mM concentration, tailor the range by down-scaling from this concentration.

NOTE: Different levels of natural compound dilutions might need to be tested (i.e., 2-fold, 5-fold or something else), depending on the natural compounds and parasites to be used in the experiments; Consequently, possibly several experiments will have to be conducted to determine the proper range of concentrations of each natural compound to be tested; It is suggested to prepare the dilutions on a log scale might work the best.

- Prepare the serial dilutions of natural compounds in filtered and autoclaved sea water;

- Fill 6-well plates with 2 mL of each dilution of natural compounds, in triplicate (if sufficient number of parasites can be collected ideally prepare 6 replicates of each concentration);

- Place 10 parasites in each well and check them under a stereomicroscope for visible signs of mechanical damage;

- Incubate the parasites in dark at room temperature for 4 hours post exposure;

- Record the number of live/dead parasites after the end of the experiment;

- Create the dose-response curves by plotting the percentage of parasite mortality against each concentration of each natural compound through an appropriate model;

- Calculate LD20, LD50 and LD80 of each natural compound from the dose-response curves.

Be sure to also include in each test:

a. NATURAL COMPOUND STERILITY CONTROL: wells with filtered and autoclaved sea water and natural compounds without parasites, in order to exclude the contamination of the tested natural compounds;
b. SEA WATER STERILITY CONTROL: wells with filtered and autoclaved sea water only, in order to exclude the contamination of medium;
c. REFERENCE ANTIPARASITIC SUBSTANCE: wells containing a reference antiparasitic substance diluted in filtered and autoclaved sea water to appropriate final concentrations and parasites, in order to compare its antiparasitic activity with that of the natural compounds;
d. PARASITE CONTROL: wells with parasites and filtered and autoclaved sea water, in order to verify their vitality at the experimental conditions;
e. SOLVENT EFFECT CONTROL (negative control): wells containing parasites and the solvent used for the preparation of natural compounds (if different from water and therefore potentially toxic to parasites) to appropriate final concentrations in filtered and autoclaved sea water, in order to exclude its potential inhibitory effects on the fish pathogen.

NOTE: The drc. package for R can be used in order to plot the dose-response curves by applying the appropriate model (log logistic, Weibull…).

IN VITRO IMMUNOMODULATORY PROPERTIES OF A PANEL OF NATURAL COMPOUNDS ON FISH HEAD KIDNEY LEUKOCYTES

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REAGENTS

Dimethyl sulfoxide (DMSO) - Merck Life Science s.r.l (MI, Italy)

Heat inactivated foetal calf serum (FCS) - Merck Life Science s.r.l (MI, Italy)

Hank's Balanced Salt Solution without phenol red, Ca2+ and Mg2+ (HBSS) - Merck Life Science s.r.l (MI, Italy)

Heparin (5 KU/ml) - Merck Life Science s.r.l (MI, Italy)

Histopaque®1077 - Merck Life Science s.r.l (MI, Italy)

Histopaque® 1119 - Merck Life Science s.r.l (MI, Italy)

L-glutamine - Merck Life Science s.r.l (MI, Italy)

Leibovitz medium (L-15) - Merck Life Science s.r.l (MI, Italy)

Penicillin/streptomycin solution - Merck Life Science s.r.l (MI, Italy)

Phorbol myristate acetate (PMA) - Merck Life Science s.r.l (MI, Italy)

Phytohemagglutinin from *Phaseolus vulgaris* (PHA-P) - Merck Life Science s.r.l (MI, Italy)

Sodium chloride (NaCl) - Merck Life Science s.r.l (MI, Italy)

Tricaine methanesulfonate (MS-222) - Merck Life Science s.r.l (MI, Italy)

Trypan blue - Merck Life Science s.r.l (MI, Italy)

3-[4,5-dimethlythiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) - Merck Life Science s.r.l (MI, Italy)

5-amino-2,3-dihydro-1,4-pathalazinedione (luminol) - Merck Life Science s.r.l (MI, Italy)

Natural compounds (i.e. MNPs, AMPs, probiotic supernatants, medicinal plant extracts or active metabolites)

FISH

Healthy fish with an adequate weight should be maintained in the experimental tanks being part of an indoor freshwater/sea water recirculating/open system supplied by with filtered and UV-treated sea water/fresh water for fish rearing and infection. They have not been submitted to previous vaccination or immunostimulation or pharmacological procedures and they should be preventively examined for their health status. They should be acclimatized to the experimental conditions for at least 14 days and fed with a species specific basal diet according to their growth. Water quality should be monitored during the experiments [temperature, pH, dissolved oxygen, NH4-N, NO_2-N should be constantly monitored]. The fish average body weight should be measured before each tissue sampling.

Fish are kept under veterinary control during the trial and any clinical sign of disease or mortality is registered. All the experimental procedures should be carried out according to the Guidelines of the European Directive 2010/63/EU on the protection of animals used for scientific purposes. Statistics should be used as tool in order to reduce the number of fish while maintaining the statistical relevance of the data.

LEUKOCYTE PURIFICATION (Galeotti *et al*. 2013)

- Euthanize fish (n=6, suitable mean weight 150-200 g) through an overdose of MS-222 (300 mg/l);
- Collect blood from the caudal vein, in order to remove it from organs as much as possible;
- Aseptically remove the head kidney and gently press it in sterile isosmolar HBSS with 0.25% heparin by a syringe piston (Fig. 4);
- Collect the cell suspension in sterile tubes and keep it on ice until the deposit of large tissue fragments on the bottom;
- Collect the cell suspension only in other sterile tubes;

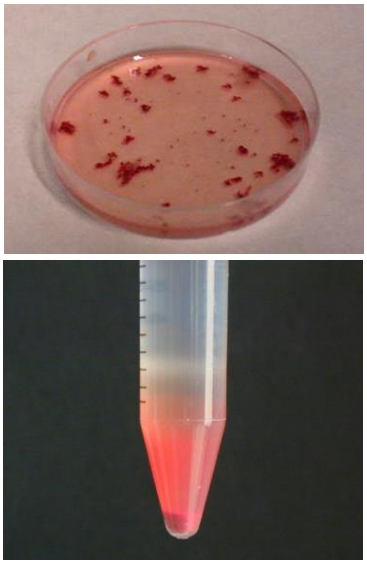

Fig 4. Sea bass head kidney fragments in isosmolar HBSS with heparin (upward) and purified leukocytes at the Histopaque® interface.

- Rinse the cells 2 times with isosmolar HBSS with 0.25% heparin by centrifugation at 210*xg* for 10 min;
- Layer the cell suspension over a discontinuous Histopaque® gradient (5ml of 1.119 density + 5ml of 1.077 density) and centrifuge at 480×*g* for 25 min at 4 °C (this protocol can be applied on sea bass cells, but it must be adapted to the fish species used in the experiments);
- Collect the leukocytes at the Histopaque® interface and transfer them in clean tubes (Fig. 4);
- Rinse the leukocytes 2 times with isosmolar HBSS without heparin by centrifugation at 210*xg* for 10 min;
- Resuspend the leukocytes in isosmolar HBSS without heparin and count the cells using the trypan blue exclusion method and Thoma chamber, according to the appropriate formula;
- Keep the leukocytes on ice until assay performance, which must be carried out in the same day of the cell purification to avoid their death.

LEUKOCYTE PROLIFERATION (Galeotti *et al.*, 1999)

DAY 1 – MICROPLATE PREPARATION AND CELL INCUBATION WITH NATURAL COMPOUNDS

- Adjust the concentration of leukocytes to 5 × 10^6 cells/ml in isosmolar L-15 containing 2 mM L-glutamine, 10% FCS, penicillin 100 U/ml, and streptomycin 100 µg/ml (culture medium);
- Add the cells (100 µl/well) into flat-bottom 96-well culture microplates;
- Incubate the cells with serial dilutions of natural compounds in isosmolar culture medium at 23 ± 2 °C in an incubator under atmospheric air for 1 h.

Be sure to also include in each test:

a. CELL VIABILITY CONTROL: untreated cells incubated with culture medium only, in order to verify the vitality of leukocytes at the experimental conditions;

b. SOLVENT EFFECT CONTROL: cells incubated with serial dilutions of the solvent used for the preparation of natural compounds (if different from water and therefore potentially toxic to fish leukocytes), in order to exclude the negative or positive effect of solvent on the leukocyte activity.

DAY 2 – DEVELOPMENT OF MTT COLORIMETRIC ASSAY

- After 1 h incubation, centrifuge the microplates at 300×*g* for 5 min and remove the supernatant;
- Incubate the adherent cells with 100 µl/well of PHA-P mitogen (50 µg/ml) in isosmolar culture medium at 23 ± 2 °C in an incubator under atmospheric air for 72-96 h;
- After the exposure, remove the medium;
- Add 40 µl/well of MTT at a concentration of 1 mg/mL;
- Incubate the microplates at 25 ± 2 °C in an incubator under atmospheric air for 4 h;
- Solubilize the MTT crystals with the addition of 150 µl/well of 100% DMSO (Fig. 5);
- Read the OD at 570 nm by using a microplate reader (i.e. Tecan Sunrise™, MI, Italy);
- Calculate the OD of leukocytes by subtracting the background (negative control) from the measured values.

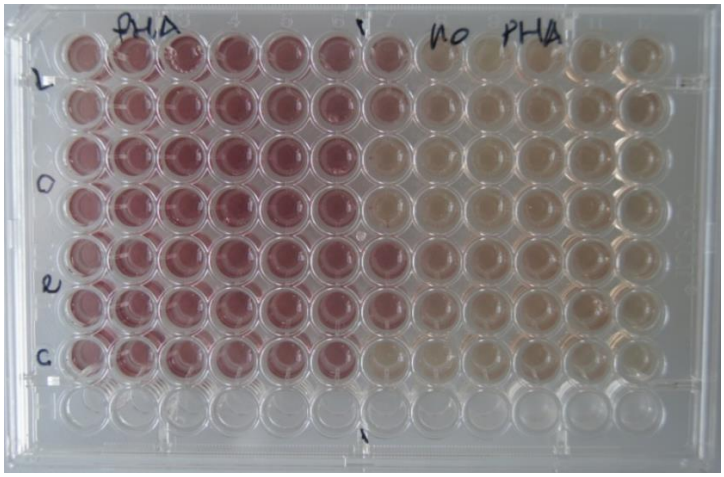

Fig. 5. Examples of microplates used for the development of the MTT assay for the evaluation of leukocyte proliferation after exposure to natural compounds, in the presence of absence of PHA-P mitogen.

Be sure to also include in each test:

a. CELL PROLIFERATION CONTROL (POSITIVE CONTROL): cells not previously exposed to natural compounds but incubated with PHA-P, in order to verify the effective proliferation of leukocytes after stimulation with PHA-P at the experimental conditions;

b. NEGATIVE CONTROL: cells not exposed to natural compounds nor to PHA-P but incubated with culture isosmolar medium only, in order to verify the basal proliferation of leukocytes at the experimental conditions in absence of stimuli and exclude the background.

LEUKOCYTE RESPIRATORY BURST (Galeotti *et al*. 2013)

MICROPLATE PREPARATION AND CELL INCUBATION WITH NATURAL COMPOUNDS

- Adjust the concentration of leukocytes to 5 × 10^6 cells/ml in isosmolar HBSS;

- Plate the cells (100 µl/well) into flat-bottom 96-well culture microplates;

- Incubate the cells with serial dilutions of natural compounds in isosmolar HBSS in an incubator at 25± 2 °C under atmospheric air for 1 h.

Be sure to also include in each test:

a. CELL VIABILITY CONTROL: untreated cells incubated with culture medium only, in order to verify the vitality of leukocytes at the experimental conditions;
b. SOLVENT EFFECT CONTROL: cells treated with serial dilutions of the solvent used for the preparation of natural compounds (if different from water and therefore potentially toxic to fish leukocytes), in order to exclude the negative or positive effect of solvent on the leukocyte activity.

DEVELOPMENT OF CHEMILUMINESCENCE (CL) ASSAY

- After 1 h incubation, centrifuge the microplates at 300×*g* for 5 min and remove the supernatant;

- Incubate the adherent cells with 100 µl/well of isosmolar HBSS, 50 µl/well of 2 mM luminol in isosmolar HBSS, and 100 µl/well of PMA in isosmolar HBSS (10 µg/ml);

- Measure the CL emission in terms of relative luminescence units (RLU) using a luminometer (i.e. Tecan s.r.l., MI, Italy) every 5-min interval for 60 min (T = 23 °C, integration time = 0.5 s, photomultiplier gain = 180);

- Calculate the luminescence of sea bass leukocytes by subtracting the background (negative control) from the measured values.

Be sure to also include in each test:

a. CELL RESPIRATORY BURST CONTROL (POSITIVE CONTROL): cells not previously exposed to natural compounds but incubated with PMA and luminol, in order to verify the effective respiratory burst activity of leukocytes after stimulation with PMA at the experimental conditions;

b. NEGATIVE CONTROL: cells not exposed to natural compounds nor to PMA but incubated with isosmolar HBBS and luminol, in order to verify the basal respiratory burst activity of leukocytes at the experimental conditions in the absence of stimuli and exclude the background.

Note: the isosmolar HBSS is a solution with the same osmolarity of fish blood: in the case of freshwater fish species, the isosmolar HBSS correspond to the commercial HBSS; in the case of seawater fish species, the isosmolar HBSS is obtained by adding an adequate quantity of NaCl to the commercial HBSS.

Examples:

European sea bass

to obtain 100 ml of isosmolar HBSS (360 mOsm/kg) mix 96 ml of HBSS with 4 ml of 20%NaCl

Gilthead sea bream

to obtain 100 ml of isosmolar HBSS (300 mOsm/kg) mix 98.25 ml of HBSS with 1.75 ml of 20%NaCl

IN VITRO CITOTOXICITY OF A PANEL OF NATURAL COMPOUNDS ON SAF-1 CELL LINE

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REAGENTS

Dimethyl sulfoxide (DMSO) - Merck Life Science s.r.l (MI, Italy)

Hank's Balanced Salt Solution without phenol red, Ca2+ and Mg2+ (HBSS) - Merck Life Science s.r.l (MI, Italy)

Heat inactivated foetal calf serum (FCS) - Merck Life Science s.r.l (MI, Italy)

L-glutamine - Merck Life Science s.r.l (MI, Italy)

Leibovitz medium (L-15) - Merck Life Science s.r.l (MI, Italy)

Penicillin/streptomycin solution - Merck Life Science s.r.l (MI, Italy)

Trypsin-EDTA solution - Merck Life Science s.r.l (MI, Italy)

Tryton™ X 100 - Merck Life Science s.r.l (MI, Italy)

3-[4,5-dimethlythiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) - Merck Life Science s.r.l (MI, Italy)

Natural compounds (i.e. MNPs, AMPs, probiotic supernatants, medicinal plant extracts or active metabolites)

CELL LINE

SAF-1 (ECACC - 00122301) fibroblast-like cell line derived from *Sparus aurata* L. caudal fin

CULTURE CONDITIONS

Cells are cultivated Leibovitz-15 medium (L-15) supplemented with 15% FCS, 2% 200mM L-glutamine and 1% penicillin/streptomycin solution (complete L-15), in 75cm^2 flasks (Sarstedt, Trezzano sul Naviglio, MI, Italy) and maintained in an incubator at 25 ± 2 °C under atmospheric air, until they reach confluence and can be used for the test.

MTT VIABILITY ASSAY (Almeida *et al.*, 2019 with some modifications)

DAY 1 - MICROPLATE PREPARATION

- Remove the culture medium from the flask and wash quickly with Trypsin-EDTA solution (3ml);
- Add Trypsin-EDTA solution (6ml), monitor under a light microscope till the complete cell detachment;
- Block the trypsin action with 24 ml of complete L-15;
- Centrifuge the cells at 130 *x g* for 10 minutes at 25 ± 2 °C;
- Count the cells under a light microscope using a Thoma chamber, according to the appropriate formula;
- Suspend the cells at a concentration of 1.25 x 10^5 cell/ml;
- Add the cells in flat-bottom 96-well culture microplates (200 µl/well);
- Allow the cells to adhere overnight in an incubator at 25 ± 2 °C under atmospheric air.

DAY 2 - CELL EXPOSURE TO NATURAL COMPOUNDS

- Examine the microplates under a light microscope to ensure the cell attachment;
- Remove the culture medium from each well and replace it with 100 µl/well of fresh complete L-15;
- Add serial dilutions of natural compounds (100 µl/well, in triplicate), previously prepared in complete L-15 using another microplate;
- Incubate the microplates in an incubator at 25 ± 2 °C under atmospheric air for 24 h.

Be sure to also include in each test:

a. CELL VIABILITY CONTROL: untreated cells incubated with culture medium only, in order to verify the vitality of cells at the experimental conditions and exclude the background;
b. SOLVENT EFFECT CONTROL: cells incubated with serial dilutions of the solvent used for the preparation of natural compounds (if different from water and therefore potentially toxic to fish cells), in order to exclude the negative or positive effect of solvent on the cell line viability;
c. POSITIVE CONTROL: cells treated with Triton™ X-100 diluted in culture media at concentrations from 5.96x10^{-8} up to 1%, in order to produce a control cytotoxic effect on the cells;
d. BLANK WELLS: wells with culture medium only but without cells.

DAY 3 – DEVELOPMENT OF MTT COLORIMETRIC ASSAY

- Remove the culture medium and wash the cells with HBSS (200 µl/well);
- Add MTT at a concentration of 0.5 mg/mL in HBSS (40 µl/well);
- Incubate the microplates in an incubator at 25 ± 2 °C under atmospheric air for 4 h;
- Solubilize the MTT crystals with 100% DMSO (150 µl/well) (Fig 6);
- Read the OD at 570 nm (reference wavelength = 690 nm) by using a microplate reader (i.e. Tecan Sunrise™, MI, Italy).
- The cell viability is expressed as percentage of the viability of cells not exposed to the natural compounds (negative control) representing the 100% viability.

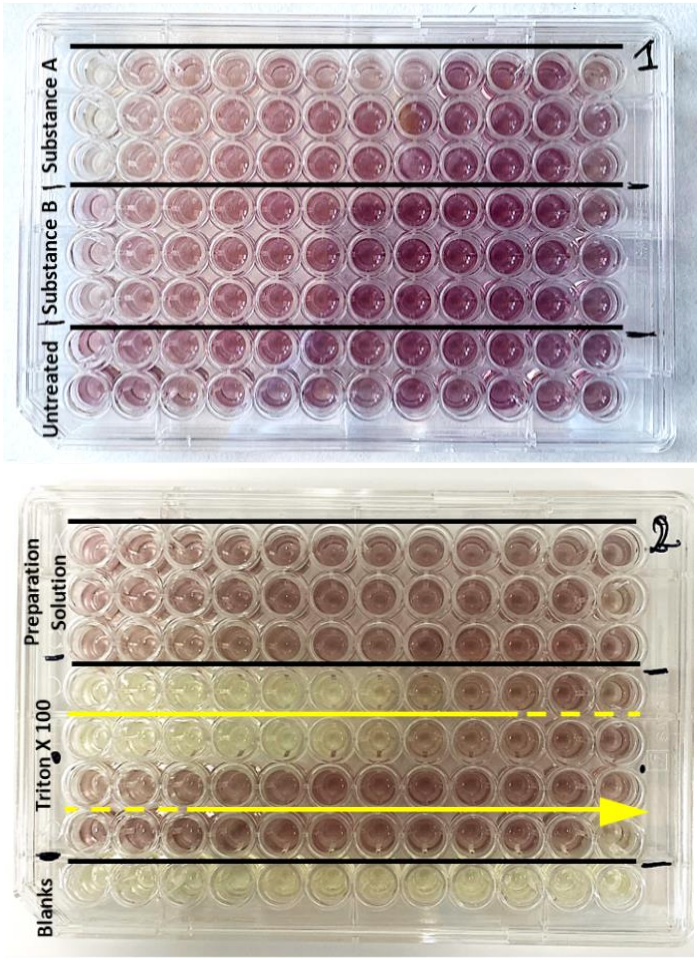

Fig. 6. Examples of microplates used for the MTT viability assay on SAF-1 cell line pre-exposed to natural compounds.

IN VIVO TRIAL FOR THE EVALUATION OF THERAPEUTIC EFFECT OF NATURAL COMPOUNDS AGAINST EXPERIMENTAL BACTERIAL INFECTION IN FISH

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REAGENTS

Culture broth and agar suitable for the selected bacterial strain (i.e. trypticase soy broth or agar)

Oxytetracycline - Merck Life Science s.r.l (MI, Italy)

Phosphate buffer saline (PBS) pH 7.2

Sodium chloride (NaCl) - Merck Life Science s.r.l (MI, Italy)

Tricaine methanesulfonate (MS-222) - Merck Life Science s.r.l (MI, Italy)

Selected natural compound

PREPARATION OF EXPERIMENTAL DIETS

The experimental diets (control negative diet, diets containing the natural compound at one or different concentrations and control positive medicated diet containing the reference antibiotic) are obtained by adding the selected natural compound or reference antibiotic at different rates (0.0% in the negative control diet and test concentrations in the supplemented diets) to a basal pelleted feed for the fish species (Tab. 1). For each diet, the amount of natural compound/oxytetracycline for 1 kg is diluted in the solvent use for the preparation of natural compound and sprayed onto or mixed with the pellets in a drum mixer. The negative control diet (0.0% natural compound) is obtained by spraying/mixing the same volume of solvent without natural compound onto/with the basal diet. Pellets are dried in a heater at 25±2°C for 48 h and stored at 4 °C in plastic bags under vacuum condition until use.

FISH AND EXPERIMENTAL DESIGN

Healthy fish with an adequate weight should be randomly allotted in the experimental tanks being part of an indoor freshwater/sea water recirculating/open system supplied by with filtered and UV-treated sea water/fresh water for fish rearing and infection. They have not been submitted to previous vaccination or immunostimulation or pharmacological procedures and they should be preventively examined for their health status. They should be acclimatized to the experimental conditions for at least 14 days and fed with a species specific basal diet according to their growth. Water quality should be

monitored during the feeding trial [temperature, pH, dissolved oxygen, NH4-N, NO_2-N should be constantly monitored].

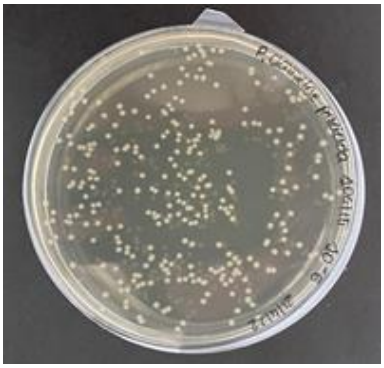
The fish average body weight should be measured before starting the trial. Then, they should be grouped and assigned to the experimental diets according to a completely random design with triplicate tanks per dietary treatment (an adequate number of fish should be allocated in each tank) (Tab. 1). Fish are hand-fed the experimental diets at the chosen ration in two meals administered at 9:00 and 16:00 hours 6 days per week, over the duration of the dietary treatment trial.

Fish are kept under veterinary control during the trial and any clinical sign of disease or mortality is registered. All the experimental procedures should be carried out according to the Guidelines of the European Directive 2010/63/EU on the protection of animals used for scientific purposes. Statistics should be used as tool in order to reduce the number of fish while maintaining the statistical relevance of the data.

GROWTH AND PREPARATION OF BACTERIA

- Inoculate the bacterial strain of interest in suitable culture broth;
- Growth the bacteria overnight at a temperature optimal for the bacterial strain, until the logarithmic phase of growth;
- Collect the bacteria after centrifugation at 2000*xg* for 20 min;
- Suspend the bacteria in sterile PBS pH 7.2 or physiological solution;
- Standardize the concentration of each strain by spectrophotometrically adjusting the optical density (OD) of the bacterial suspension at wavelength = 610 nm to 1.0 (based on experimental data, this OD value correspond to a concentration of 1×10^9 CFU/ml for some of the common fish bacterial pathogens) by using a microplate reader (i.e. Tecan Sunrise™, MI, Italy);
- Dilute the bacteria in sterile PBS or physiological solution, in order to reach the appropriate bacterial concentration;
- Spread 50 µl of 10^5 and 10^6 dilutions of bacterial suspension onto TSA plates (in duplicate);
- Incubate the plates at an optimal temperature for the fish pathogen for 24 h;

- After incubation, count the number of colony forming units (CFU), in in order to confirm the bacterial concentration (Fig. 7).



Note: preliminary experimental infections should be performed in a small number of control fish using at least 3 different doses of bacteria, in order to determine the lethal dose 70 (DL70, Council of Europe, 2010) to be used in the definitive experimental infection.

Fig. 7. Bacterial colonies on culture agar.

EXPERIMENTAL INFECTION (CHALLENGE) AND TREATMENT

- Anesthetize at least 40 fish per dietary treatment with an appropriate dose of MS-222 (dose based on the fish weight);
- Intraperitoneally inject 100 µl/fish of infective dose (preliminary test doses or DL70) in at least 30 fish per dietary treatment;
- Re-allocate the fish into the three replicate tanks per dietary treatment (10 fish/tank) supplied with filtered and UV-treated sea water/fresh water (open system) (Fig. 8);
- Intraperitoneally inject 100 µl/fish of physiological solution or PBS in a further group of 10 fish per dietary treatment as non-infected controls;
- Allocate the fish into one tank per dietary treatment (10 fish/tank) supplied with filtered and UV-treated sea water/fresh water (open system);
- Start to fed fish with the experimental diets one day after infection;
- Monitor the fish mortality for the subsequent 10-14 days, with periodical controls every 2 h during the first two days and then every 12 h;
- Sacrifice the moribund fish with a lethal dose of MS-222 in seawater (based on fish weight, commonly up to 300 mg/l);
- Examine the dead fish for clinical signs of disease (necroscopy evaluation);
- Re-isolate the bacteria from organs, in order to confirm the cause of fish mortality;

- Describe the kinetic of fish mortality as suggested by Kurath (2008);
- Calculate the percentage of cumulative mortality (Nordmo, 1997) and express the protection conferred by the natural compound as a relative percentage of survival (RPS), as proposed by Amend (1981) for the evaluation of vaccine efficacy:

RPS = [1- (% mortality in treated group /% mortality in control non treated group)] x 100

Fig. 8. Experimental tanks being part of an indoor freshwater/sea water recirculating/open system supplied with filtered and UV-treated sea water/fresh water for fish rearing and infection at the University of Udine.

IN VIVO TRIAL FOR THE EVALUATION OF IMMUNOSTIMULANT EFFECTS OF NATURAL COMPOUNDS IN FISH

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REAGENTS

Acetic acid 30%

Bovine serum albumin (BSA)

Bradford reagent - Merck Life Science s.r.l (MI, Italy)

Culture broth and agar suitable for the selected bacterial strain

Ethylene glycol bis (2-aminoethyl ether) N, N, N ', N'-tetracetic acid (EGTA) - Merck Life Science s.r.l (MI, Italy)

Hank's Balanced Salt Solution without phenol red, Ca2+ and Mg2+ (HBSS) - Merck Life Science s.r.l (MI, Italy)

Hydrogen peroxide (H_2O_2)- Merck Life Science s.r.l (MI, Italy)

Lysozyme from chicken egg white- Merck Life Science s.r.l (MI, Italy)

Magnesium chloride ($MgCl_2$ ($6H_2O$))

Micrococcus luteus- Merck Life Science s.r.l (MI, Italy)

Nα-benzoyl-DL-arginine-p-nitroanilide (BAPNA) - Merck Life Science s.r.l (MI, Italy)

Phosphate buffer saline (PBS) pH 7.2

Polyethylene glycol (PEG) - Merck Life Science s.r.l (MI, Italy)

Sheep red blood cells

Sodium barbiturate- Merck Life Science s.r.l (MI, Italy)

Sodium chloride (NaCl) - Merck Life Science s.r.l (MI, Italy)

Sodium phosphate ($Na_2HPO_{4)}$

Sulphuric acid (H_2SO_4) - Merck Life Science s.r.l (MI, Italy)

3, 3 ', 5.5' - tetramethylbenzidine (TMB) - Merck Life Science s.r.l (MI, Italy)

Tricaine methanesulfonate (MS-222) - Merck Life Science s.r.l (MI, Italy)

Trypsin from bovine pancreas- Merck Life Science s.r.l (MI, Italy)

Tris-HCl- Merck Life Science s.r.l (MI, Italy)

Selected natural compound

PREPARATION OF EXPERIMENTAL DIETS

The experimental diets (control negative diet, diets containing the natural compound at one or different concentrations and positive control diet containing a standard immunostimulant) are obtained by adding the selected natural compound or standard immunostimulant at different rates (0.0% in the control diet and test concentrations in the supplemented diets) to a basal pelleted feed for the fish species (Tab. 1). For each diet, the amount of natural compound or standard immunostimulant for 1 kg is diluted in the solvent use for the preparation of natural compound and sprayed onto or mixed with the pellets in a drum mixer. The control diet (0.0% natural compound) is obtained by spraying/mixing the same volume of solvent without natural compound or standard immunostimulant onto/with the basal diet. Pellets are dried in a heater at 25±2°C for 48 h and stored at 4 °C in plastic bags under vacuum condition until use.

FISH AND EXPERIMENTAL DESIGN

Healthy fish should be obtained from a local fish farm when they have an adequate weight and examined for their health status immediately upon arrival. They have not been submitted to previous vaccination or immunostimulation or pharmacological procedures.

Fish should be randomly allotted in the experimental tanks being part of an indoor freshwater/sea water recirculating/open system supplied by with filtered and UV-treated sea water/fresh water for fish rearing and infection. They should be acclimatized to the experimental conditions for at least 14 days. Water quality should be monitored during the feeding trial [temperature, pH, dissolved oxygen, NH4-N, NO_2-N should be constantly monitored].

The fish average body weight should be measured before starting the feeding trial. Then, they should be grouped and assigned to the experimental diets according to a completely random design with triplicate tanks per dietary treatment (an adequate number of fish should be allocated in each tank) (Tab. 1). Fish

are hand-fed the experimental diets at a fixed ration in two meals administered at 9:00 and 16:00 hours 6 days per week, over the duration of the feeding trial. At the end of the feeding trial, fish should be group-weighted after 48-h fast and under sedation with MS-222.

Fish are kept under veterinary control during the trial and any clinical sign of disease or mortality is registered. All the experimental procedures should be carried out according to the Guidelines of the European Directive 2010/63/EU on the protection of animals used for scientific purposes. Statistics should be used as tool in order to reduce the number of fish while maintaining the statistical relevance of the data.

BLOOD AND TISSUE SAMPLING

At least two blood sampling should be performed during the feeding trial (i.e. after 4 week of feeding and at the end of the trial), in order to evaluate the humoral and cellular immune parameters:

- After 24 h- fasting, randomly take at least 3 fish/tank with a clean net;

- Sedate fish by immersion in MS-222 (dose based on the fish weight);

- Collect blood from the caudal vein by using a syringe with an adequate volume;

- Centrifuge the blood sample at 1500*xg* for 15 min after coagulation on ice, in order to obtain serum;

- Store the serum samples at -80 ° C until use;

- Euthanize fish with a lethal dose of MS-222 in seawater (based on fish weight, commonly up to 300 mg/l);

- Open the abdominal cavity of fish with clean pliers and scissors, and collect head kidney;

- Preserve the tissue in isosmolar HBSS on ice until use.

SERUM TOTAL PROTEINS (Bradford 1976)

- Prepare two-fold serial dilutions of serum (40 µl /well) in flat bottom 96-well microplates;
- Prepare two-fold serial dilutions of BSA (1 mg/ml) as positive control;
- Prepare some wells containing distilled water only as blank;

- Add 200 µl/well of Bradford reagent;
- Measure the OD at 595 nm using a microplate spectrophotometer (i.e. Tecan Sunrise™, Milan, Italy);
- Calculate the serum total protein concentration (mg/ml) from the calibration curve of BSA.

SERUM TOTAL IMMUNOGLOBULINS (Contessi et al. 2006)

- Incubate 100 µl of serum with 100 µl of 12% PEG solution (10 000 kDa) in eppendorf at room temperature (RT) in constant shaking for 2 h;

- Centrifuge the samples at 5000*x g* for 15 min;

- Collect the serum supernatant;

- Submit the supernatant to total protein quantification as described above;

- Calculate the serum total IgM concentration (mg/ml) as the difference between the serum total protein concentration and the protein concentration measured in the corresponding supernatant.

SERUM COMPLEMENT ACTIVITY (Contessi et al. 2006, partially modified)

- Dilute the fish serum from 1:8 to 1:40 in 0.1 M EGTA-Mg-GVB in adequate tubes (final volume 100 µl, in triplicate);
- Prepare tubes containing 100 µl of EGTA-Mg-GVB as negative blank control (in triplicate);
- Prepare tubes containing 100 µl of distilled water as positive haemolysis control (in triplicate);
- Add 25 µl of 1% sheep red blood cells ($2x10^8$ red cells / ml) in EGTA-Mg-GVB
- Incubate the samples at 22-23 ° C for 100 min with constant shaking;
- After incubation, stop the reaction by adding 1 ml/tube of cold 60 mM PBS + EDTA;
- Centrifuge the tubes at 1250*xg* for 5 min at 22 °C.;
- Visually assess the lysis of sheep RBC based on the absence/presence and size of the RBC pellet in the tube, then read the DO at 415 nm using a microplate reader (i.e. Tecan Sunrise™, Milan, Italy) after transferring 200 µl of supernatant to flat-bottom 96-well plates.

The reciprocal of the serum volume which induces the lysis of 50% of the sheep's red cells is designated as ACH50. The results are presented as U/ml.

SERUM ANTIPROTEASE ACTIVITY (Bowden et al. 1997, partially modified)

- Dilute the fish serum from 1:5 to 1:20 in 0.01 M Tris-HCl pH 8.2;
- Incubate 10 µl of the diluted serum with 10 µl of 0.5% trypsin in 0.01 M Tris-HCl pH 8.2 in adequate tubes (in triplicate);
- Prepare tubes containing 10 µl of 0.01 M Tris-HCl pH 8.2 and 10 µl of trypsin in 0.01 M Tris-HCl pH 8.2 as positive control (in triplicate);
- Prepare tubes containing 20 µl of 0.01 M Tris-HCl pH 8.2 only as negative control (in triplicate);
- Incubate the samples for 5 min at 22 ± 2 °C;
- Add 500 µl of BAPNA in each tube;
- Add 480 µl of 0.1 M Tris-HCl pH 8.2 in each tube;
- Incubate the samples at 22 ± 2 °C for 25 min;
- After incubation, stop the reaction by adding 150 µl of 30% acetic acid in each tube;
- Centrifuge the samples at 400*xg* for 5 min;
- Collect the supernatant and transfer it into flat-bottom 96-well microplates;
- Read the OD at 410 nm using a microplate spectrophotometer (i.e. Tecan Sunrise™, Milan, Italy);
- Calculate the activity of serum antiproteases as percentage of trypsin inhibition (Zuo and Woo, 1997):

Percentage of trypsin inhibition (%) = [(O.D. trypsin - O.D. sample)/O.D. trypsin] x 100

SERUM PEROXIDASE ACTIVITY (Quade and Roth 1997, partially modified)

- Incubate 15 µl/well of serum with 135 µl/well of HBSS in flat bottom 96-well microplates (in triplicate);
- Prepare wells containing 150 µl of HBSS as negative control (in triplicate);
- Add 50 µl/well of 2 mM TMB with 5 mM H_2O_2
- Incubate the samples for 2-5 min at RT;
- Stop the reaction by adding 50 µl/well of 2 M H_2SO_4
- Measure the OD at 450 nm using a microplate reader (i.e. Tecan Sunrise™, Milan, Italy);
- Express the serum peroxidase activity as OD value.

SERUM LYSOZYME ACTIVITY (Parry et al. 1965, partially modified)

-Incubate 10 µl/well of serum in flat bottom 96-well microplates (in triplicate);

- Prepare wells with 10 µl of 0.05 M Na_2HPO_4 pH 6.2 as negative control (in triplicate);

- Prepare wells with two-fold dilutions of lysozyme purified from chicken white eggs as positive control (in triplicate);

- Add 200 µl/well of 0.02% *M. luteus* in 0.05 M Na_2HPO_4 pH 6.2;

- Measure the decrease of turbidity in the wells due to the lysis of bacteria caused by the serum or control lysozyme during an incubation of 60 min, by reading the OD at 450 nm every 1 min during the first 10 min then every 10 min using a microplate spectrophotometer (i.e. Tecan Sunrise™, Milan, Italy);

- Calculate the serum lysozyme activity (U/ml) using the equation of the calibration line make using the readings of the dilutions of lysozyme purified from chicken eggs.

DISEASE RESISTANCE AFTER EXPERIMENTAL INFECTION (CHALLENGE) WITH A BACTERIAL PATHOGEN

A challenge experiment with a specific fish pathogen could be performed at the end of the feeding trial, in order to evaluate fish resistance to disease:

- Prepare the pathogen of interest as described in page 35.
- Anesthetize at least 40 fish per dietary treatment (10 fish/tank) by immersion in MS-222 (dose based on fish weight);
- Intraperitoneally inject 0.1 ml/fish of bacterial suspension (the infective dose should be equal to DL70 and should be selected based on preliminary infection experiments on a small number of control fish) in at least 30 fish per dietary treatment;
- Re-allocate the fish into the three replicate tanks per dietary treatment (10 fish/tank) supplied with filtered and UV-treated sea water/fresh water (open system);
- Intraperitoneally inject a further group of 10 fish per dietary treatment with 0.1 ml of physiological solution or PBS as non-infected controls;

- Allocate the fish into one tank per dietary treatment (10 fish/tank) supplied with filtered and UV-treated sea water/fresh water (open system);
- Monitor the fish mortality for the subsequent 10-14 days, with periodical controls every 2 h during the first two days and then every 12 h;
- Sacrifice the moribund fish with a lethal dose of MS-222 in seawater (based on fish weight, commonly up to 300 mg/l);
- Examine the dead fish for clinical signs of disease (necroscopy evaluation);
- Re-isolate the bacteria from organs, in order to confirm the cause of fish mortality;
- Describe the kinetic of fish mortality as suggested by Kurath (2008);
- Calculate the percentage of cumulative mortality (Nordmo, 1997) and express the protection conferred by the natural compound as a relative percentage of survival (RPS), as proposed by Amend (1981) for the evaluation of vaccine efficacy:

RPS = [1- (% mortality in treated group /% mortality in control non treated group)] x 100

Tab. 1. Tentative quantification of natural compound request for in vivo trial aimed at evaluating its potential antimicrobial or immunostimulant effect.

	THERAPY	IMMUNOSTIMULATION
Groups	**Infected (3 groups):** 1. Treated with new candidate 2. Not treated 3. Treated with reference antibiotic **Not Infected (3 groups):** 1. Treated with new candidate 2. Not treated 3. Treated with reference antibiotic	**Infected (2 groups):** 1. Treated with new candidate 2. Not treated 3. Treated with reference immunostimulant **Not Infected (2 groups):** 1. Treated with new candidate 2. Not treated 3. Treated with reference immunostimulant
Number of fish	15/tank	
Replicates tanks	3	
Fish mass	150 g/tank (10 g/fish)	
Treatment duration	7-14 days oxytetracycline as reference antibiotic	2-6 weeks
Inclusion level	35-75 mg/kg live weight/day oxytetracycline as reference antibiotic	1%-5% in feed
Feeding Rate	2.5%	2.5%
QUANTITY OF NATURAL CANDIDATE REQUESTED	**220 mg min – 945 mg max**	**3.2 g min – 47.3 g max**

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