Interreg Italy - Croatia AdriAquaNet

EUROPEAN UNION

AdriAquaNet

Enhancing Innovation and Sustainability in Adriatic Aquaculture

MID TERM TECHNICAL-SCIENTIFIC REPORT OF **WP4**

Period: 01.01.2019-30.06.2020

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The challenge

Adriatic mariculture provides highly valued fish products for both local and distant markets. This sector can further develop thanks to new available technologies and stronger information for consumers. The sector can offer high qualification job opportunities and boost local economy.

The team

Within **AdriAquaNet** project, **4 industries**, **1 consortium** and **6 research laboratories** from both **Italy** and **Croatia** are teaming up to develop and apply technologies for fish farming and marketing. This is the first ever initiative for improving the quality of fish farming and marketing by cooperation between both sides of the Adriatic Sea.

Partners

UNIVERSITÀ DEGLI STUDI DI UDINE
hic sunt futura

UNIVERSITÀ DEGLI STUDI DI TRIESTE

INSTITUT ZA OCEANOGRAFIJU I RIBARSTVO SPLIT

Istituto Zooprofilattico Sperimentale delle Venezie

SVEUČILIŠTE U RIJECI UNIVERSITY OF RIJEKA
FAKULTET ZA MENADŽMENT U TURIZMU I UGOSTITELJSTVU
FACULTY OF TOURISM AND HOSPITALITY MANAGEMENT
OPATIJA, HRVATSKA CROATIA

Consiglio Nazionale delle Ricerche
Istituto di Chimica Biomolecolare

KLASTER MARIKULTURA

FRIŠKINA d.o.o.
uzgoj ribe i školjki

ITTICA CALDOLI
SOCIETÀ AGRICOLA ARL

ORADA ADRIATIC

ROYAL ADRIATIC

Friul Trota

THE MID TERM TECHNICAL-SCIENTIFIC REPORT

It is a deliverable of WP2.2 due on 30.06.2020 (3rd report) and it was postponed to be delivered with the PR4. Its aim is to show the progress and achievements (state-of-the art) in WP3-4-5 to the project stakeholders (administrative professionals, SMEs and associations, consumers). At the end of the project, this mid-term will be refined in order to prepare the final technical-scientific report. The report will support the scientific management of the project's activities. Scientific Board and the WP leaders (LP, PP1, PP3) are responsible for the content, while LP supervises the work of all WPs. LP supervises all WPs. PP2 assists in formatting, editing and using contents for WP2.

GOAL 1
New approaches to improve fish nutrition and ensure efficient waste management

the fish farm

ACTIONS
- Implement novel feeds to improve farmed fish welfare
- Provide tailored feeding protocols to allow an innovative food management
- Provide new technologies to improve energy saving and farm environmental footprint

GOAL 2
New strategies to enhance fish health and welfare

the fish doctor

ACTIONS
- Develop new vaccines/ vaccination strategies
- Test novel probiotics/ nutraceuticals for controlling infectious diseases
- Develop easy, rapid and effective methods to assess fish welfare

GOAL 3
New fish products for different classes of consumers

the fish market

ACTIONS
- Apply new technologies to develop high-quality fish products
- Transfer knowledge to SMEs for improving the quality of fish products and their marketing
- Promote to consumers the nutritional value of farmed fish and health benefits of its consumption

WP 4
R&I TO IMPROVE HEALTH AND SUSTAINABILITY IN ACQUACULTURE

WP 4 Leader
Dr. Ivona Mladineo
Institute of Oceanography and Fisheries, Split, Croatia

1. OBJECTIVES, APPROACH & ACTIVITIES

The main goal of WP4, that is **to secure economic sustainability to the aquaculture business sector, by production of healthy, welfare-centered and consumer acceptable fish**, will be achieved by four main approaches.
Firstly, **vaccine production and development of vaccination strategies** will mitigate seabass and seabream vibriosis and tenacibaculosis, providing tailor-made formulations against these two fish bacterial pathogens for which no commercial vaccine exists.
Secondly, natural therapeutic substances/probiotics/marine **natural products**, will be identified and tested to secure new, innovative and alternative compounds to mitigate the antimicrobial resistance to conventional antibiotics or the employment of less environmentally-sustainable drugs against bacteria and parasites.
Thirdly, welfare monitoring will provide **far-reaching tools for the evaluation of health status/welfare of farmed fish**, easily and quickly applicable directly on field by farmers and veterinarians, so that both the farming management and productivity will be improved.
Finally, the **resulting new knowledge derived from the three listed approaches will be transferred to professionals and experts**, i.e fish farmers and veterinarians, in frame of three training events (Italy and Croatia), to ensure appropriate and efficient uptake of WP4 deliverables.

In the first semester, activities carried out within 4.1. were cultivation, checking, virulence analysis and choosing of bacterial isolates *(Vibrio harveyi* and *Tenacibaculum maritimum*) that will be used for the production of tailor-made vaccines (PP1, PP4). In the frame of 4.2, flowers of the traditional medical plant *Dalmatian chrysanthemum*; were collected, dried and prepared for isolation of pyrethrins (PP3); microbiota isolates from Adriatic-wide farmed seabream and seabass were collected, isolated, biochemically and molecularly identified (PP3); bibliographic research on the use of marine natural products (MNPs) as antibacterials and immunostimulants was performed (LP), the experimental

protocol for the *in vitro* antibacterial assays was standardized (LP), the MNPs to be tested were selected and provided (PP6), the bacterial strains for testing (*V. anguillarum*, *Photobacterium damselae* subsp. *piscicida* and *Ph. damselae* subsp. *damsealae*) were provided (PP4), and *in vitro* antibacterial assays were initiated (LP).
In the second semester, activities within 4.1. consisted of purity-checking, molecular typing, pathogenicity and virulence evaluation of the chosen *V. harveyi* and *T. maritimum* isolates (PP1), production of bacterial isolates in bioreactors (PP1), preparation of vaccines (PP1), and preliminary vaccine testing in experimental small-scale trial (PP1, PP3) (Figure 1, 2). Within 4.2. pyrethrins from the *Dalmatian chrysanthemum* (Figure 3) were extracted by supercritical CO_2 extraction (SFE) and characterized by HPLC (PP3); a list of potentially probiotic isolates has been developed from microbiota isolates of Adriatic-wide aquaculture fish and the probiotic candidate to be tested in the feeding trial has been identified (PP3) (Figure 4); further crude extracts and enriched fractions of NMPs were purified through solid-phase extraction (PP6) and compounds were tested by *in vitro* antimicrobial assays (LP) (Figure 5). Within 4.3. a general review of literature on Operational Welfare Indicators (OWI) was performed (PP4), a study visit to PP10 was conducted to discuss the preliminary draft of OWI (PP4), and subcontractors to be engaged in development of Bivalent Monitoring Tool and supply of photovoltic panels were chosen (PP4) (Figure 6).
In the third semester, within 4.1. ELISA protocols for the evaluation of specific antibodies titres against *V. harvey* and *T. maritimum* in seabass sera were developed (LP) and sera collected (14 days post treatment) from fish submitted to small-scale immersion and intraperitoneal vaccination trials by PP1 and PP3 were analyzed (LP), a new batch of the vaccines (monovalent V.H., monovalent T.M, bivalent V.H.+T.B.) required for the new set of vaccination trials was produced (PP1) and the trial was performed in the facility of the PP3 by intraperitoneal application and by immersion (PP1). Within 4.2. a bibliographic review on probiotics isolated from fish intestinal tract and their use as dietary supplements was performed (LP), culture supernatants of bacterial isolates from farmed seabream and seabass intestines were prepared and sent to LP to be tested for antimicrobial and immunostimulatory properties (PP3), selected isolate of *Bacillus subtilis* was further characterized as a new probiotic and sent to

Italian feed producer for incorporation in new feed formulation (PP3), the feeding trial started and probiotic feed was included in the trial starting a month later, and the first two tissues collections (TEM, IHC histology, gene expression and microbiome) were performed (PP3). In addition within 4.2., further testing to determine the antibacterial activity of new MNPs and 8 antimicrobial peptides (AMPs) provided by PP6 against *Vibrio anguillarum* O1 and *Photobacterium damselae* subsp. *piscicida* were carried out (LP), *in vitro* culture/maintenance of SAF-1 GSB cell line to be used for the *in vitro* toxicological and immune-stimulation tests optimised (LP). Blood sampling from control sea bass and sea bream maintained at LP facilities was performed (LP), and measurement of serum bilirubin/biliverdin to be used as fish blood biomarker was undertaken (PP2) (Figure 7).

2. EXPECTED RESULTS

So far all delivered results have been shown regularly during project meetings, but the disclosure of fully analyzed and meaningful results corroborated by statistical test will be performed at the end of the project. Briefly, in 4.1. **three different vaccine types (two mono and one bivalent) were tested with different outcomes**, but the titre of the circulating post-vaccination IgM still needs to be determined in order to evaluate vaccine efficiency. In 4.2. **antimicrobial activity of different NMPs has shown varying results that need to be related to cytotoxicity and immunostimulant capacity of these compounds, which still need to be tested**. The isolated probiotic used in seabream feeding trial will show the efficacy once the trial is finalized and all samples analyzed. Natural pyrethrins still need to be tested against frequent aquaculture parasites. In 4.3. **the assembly of the buoy that incorporates equipment for measurement of abiotic parameters in cages is to be finalized and the assay for measurement of bilirubin from fish serum and whole blood, as a parameter of welfare needs to be further optimized.** Use of listed welfare indicators need to be further approved by engaged partners, so the design of their testing can be developed.

3. EXPECTED IMPACT

Although all four WP4 activities are interlinked, contributing altogether to the general objective **to secure economic sustainability to the aquaculture business sector, by production of healthy, welfare-centred and consumer acceptable fish**, each one has independent protocols and life cycle, therefore acquired mid-term results have been used to build up each task separately.

In 4.1. the characterization of bacterial pathogens has enabled to choose the most pathogenic Adriatic isolates frequently conditioning mortalities in aquaculture. Their typisation and selection has contributed to the production of a preliminary vaccine that has been firstly tested in small-scale laboratory environment, then an additional batch of vaccines has been produced and prepared for testing in a second trial at PP3 facilities and in the field conditions. This valuable upscaling will evidence the real protection efficiency of vaccines in real-time environment where other abiotic traits (temperature, parasites, fed) can affect the outcome of vaccination. The expected impact after the third semester that could not be achieved without previously achieved technical milestones are the availability of at least two new vaccines that will improve fish health, thus limiting the use of antibiotics and other chemicals that cause fish farm pollution and weaken natural fish resistance to environmental and microbial stress (prospective patent application). The best candidate that will result from the screening of different MNPs will represent alternative solutions to synthetic compounds, enabling better response to therapy because of no developed antibacterial resistance of fish bacteria to new compounds, and consequently less chemical load in both fish and environment. The new NMPs will be chosen based on efficient antimicrobial activity against most common pathogens affecting aquacultured sea bass and sea bream, such as *Vibrio anguillarum*, *Photobacterium damselae* subsp*. piscicida, and Photobacterium damselae* subsp*. damselae*, as well as immunostimulatory properties towards seabass head kidney leukocytes. This bivalent efficiency will have expected impact on both the specific (against bacteria) and innate (enhanced intrinsic immunocompetence of fish) protection. To stimulate better digestion and innate immunity,

new probiotic isolate added in new feed formulation for aquaculture was developed, taking into account that autochthonous isolate from Adriatic-farmed fish would prove better efficiency than an allochthonous isolate. Addition of probiotic will strengthen innate mucosal immunity and digestibility of feed, providing beneficial effect for multiple instances; fish health, growth rate and consumer safety.

Lastly, natural pyrethrins isolated from *Dalmatian chrys*anthemum, if proven efficient against Adriatic aquaculture parasites, such as the monogenean *Sparicotyle chrysophrii* and the isopod *Ceratothoa oestrides*, will have impact in terms of decreased use of synthetic chemicals. This is very important because the most widely used compound against these parasites is formaldehyde, which has been banned for use in aquaculture in Italy, and other EU countries will likely follow the same direction. Therefore, **efficient and natural antiparasitic will greatly impact the sustainability of the current aquaculture practices, contributing to the protection of the environment and human health.** In 4.3., the development of in-farm applicable and easy to use welfare monitoring for farmers and veterinarians, will provide far-reaching tools for the evaluation of health status/welfare of farmed fish. The application of the welfare-monitoring tools, based on developed indicators specific for seabass and seabream, will have an impact in terms of improving fish farming management and productivity. Lastly, the activities developed within 4.4. will directly impact farmers' productivity and broaden expert knowledge, because technologies and outputs from above described tasks will be transferred to other SMEs and veterinarian professionals, through jointly organized training courses. It is expected that the communication activities and deriving materials will raise awareness about technology transfer opportunities.

4. PROBLEM(S)

Main problem arisen during COVID-19 lockdown that disabled any equipment acquisition, as well as laboratory and field work, resulting in lagging behind of mostly laboratory work. Fish feeding trial (4.2.) started in time as PP8 continued to work on the farm and new feeds were delivered in time, so the experiment started in time. However, some collections of samples (serum for serological analyses), was postponed for the end of lockdown. Setup of experimental trial of natural pyrethrins against aquaculture fish parasites was also postponed; partially because the proliferation of pathogens was not high in the last semester, and partially because of no possibilities to transfer the parasites to PP3 during the lockdown. The trainings were postponed to the forth semester. partially because the proliferation of pathogens was not high in the last semester, and partially because of no possibilities to transfer the parasites to PP3 during the lockdown. The trainings were postponed to the forth semester.

Figure 1
Vaccination trial of fingerlings seabass in laboratory-scale facility of PP3 (Activity 4.1.).

Figure 2
Seabass fingerlings ready to be distributed in laboratory-scale tanks at PP3 for the vaccination trial (Activity 4.1.).

Figure 3
Collection of the Dalmatian chrysanthemum flowers for the extraction of natural pyrethrins (Activity 4.2.):

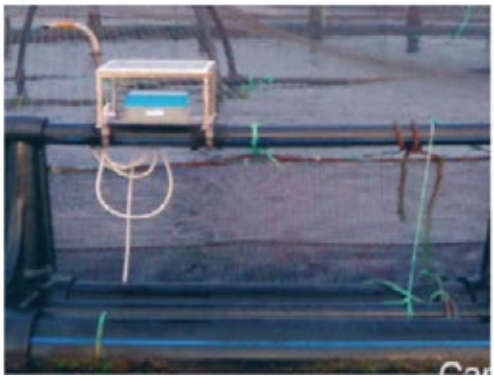
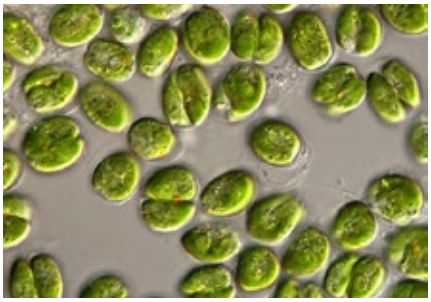


Figure 4
The equipment to monitor environmental parameters (salinity, temperature, oxygen) with video camera tested and calibrated by PP4 marine aquarium (Activity 4.3.).

Figure 5
Collection of seabream samples during the feeding trial with three different feeds, at PP8 facility (Activity 4.2.).



Tetraselmis suecica

Chondrilla nucula

Haliclona mediterranea

Buccinulum corneum

Figure 6
Some of different marine organisms, which crude extracts have been isolated and tested for antimicrobial activity and immunostimulatory potential in seabass by LP (Activity 4.2.).

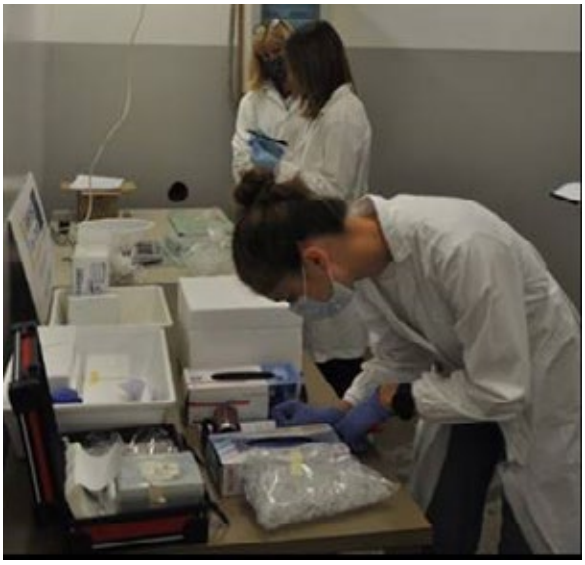
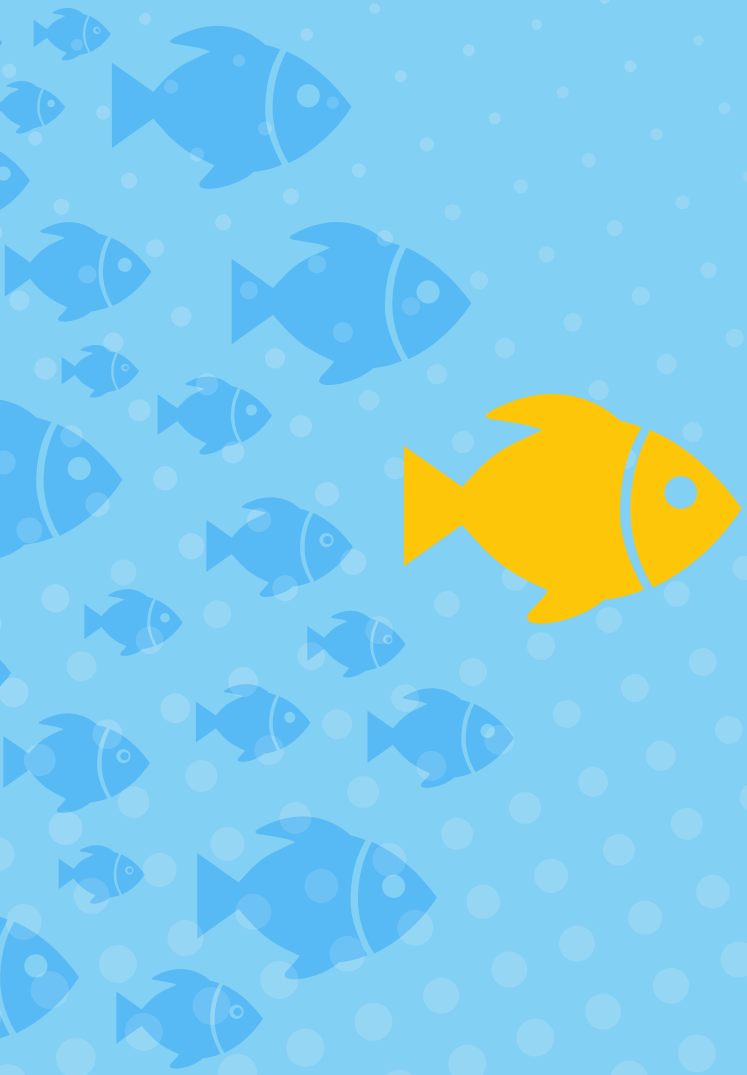


Figure 7
Staff of LP and PP2 preparing fish serum for microanalysis of bilirubin at the Azienda Agraria Antonio Servadei of the University of Udine.



CONTACT

University of Udine
Prof. Marco Galeotti
marco.galeotti@uniud.it

www.italy-croatia.eu/AdriAquaNet

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