AdriAquaNet

Enhancing Innovation and Sustainability in Adriatic Aquaculture

Deliverable WP 4.1

Autologous “tailor-made” vaccines against *Tenacibaculum maritimum*

PP1 CVI

Control sheet/Control Document

Zagreb, 30.06.2022

PART 1

Project number:	10045161
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Project Title:	Enhancing Innovation and Sustainability in Adriatic Aquaculture
Start of the project:	01/01/2019
Duration:	42 months
WP/activity:	WP4/Vaccine production and vaccination strategy
Deliverable name:	4.1. Autologous "tailor-made" vaccines against *Tenacibaculum maritimum*
WP leader:	Snježana Zrnčić
Author (s):	Snježana Zrnčić and Dražen Oraić
Delivery date:	30.07.2022.
Status:	**Final**

1) *Introduction (objective and purpose of the deliverable)*

Infection caused by Gram (-) negative, filamentous bacteria, 0.4-0.5 µm in diameter and 1.5-30 µm, sometimes up to 100 µm long bacterium *Tenacibaculum maritimum* (Fig. 1) is considered as one of the most important diseases of European seabass (*Dicentrarchus labrax*) (Zrnčić and Pavlinec, 2020).

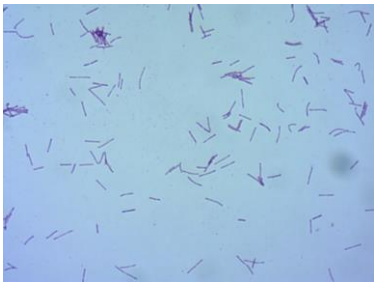

Figure 1. Gram-stained smear of pure bacterial colony of *T. maritimum*

Affected fish are lethargic, show loss of appetite, hemorrhagic and necrotic lesions on the skin around the eyes, on the head, eroded mouth, and lesions such as frayed fins and tail rots and in some cases necrosis on the gills and eyes (Toranzo et al., 2015; Piñeiro-Vidal et al., 2007). The lesions are characterized by increased mucus production and presence of whitish necrotic tissues (Smage et al., 2016). Fish with gill infections display an increased respiratory rate (Mitchell and Rodger, 2011) with yellow or brown mats on the pale gills, and extensive areas of severe necrosis (Fig. 2-5). Deeper ulcerative lesions on the body sides have been reported (Le Breton et al., 2018). Once disease affects farmed sea bass it causes high mortalities and significant economic losses (Castro et al., 2014).
Croatian marine fish farms are faced with difficulties in managing outbreaks of tenacibaculosis in sea bass which very often affects younger categories (Zrnčić et al., 2013). Losses could be mitigated by controlling fish density, reducing stress, avoiding overfeeding and by administration of antibacterial drugs such as tetracyclines, potentiated sulphonamides and fluoroquinolones (Avendano-Herrera et al. 2006). However, very often field results are not coherent with in results of in vitro testing of susceptibility. There is a single commercial vaccine for turbot in Spain

prof. Marco GALEOTTI DVM, Dipl. E.C.V.P.
University of Udine
Department of Agricultural, Food, Environmental and Animal Sciences
Via Sondrio, 2, Udine, 33100, Italy
+39 0432-558594
marco.galeotti@uniud.it

(Romalde et al., 2005), and several autologous vaccines against tenacibaculosis were tested for different marine fish species as for barramundi in Singapore (Rafidah et al., 2015), salmon in Tasmania (Van Gelderen et al., 2009) or salmon in Chile (Tobar, 2015), so far.

Figure 2. Hemorrhagic and necrotic lesions on the skin

Figure 3. Gill necrosis with yellow mats

Figure 4. Frayed fins

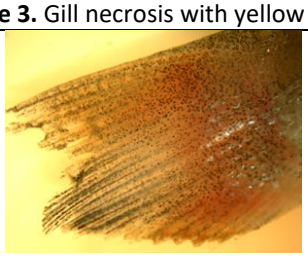

Figure 5. Tail rot

Aiming at improve fish health in the Adriatic aquaculture and contribute to its sustainability and to the one health by decreasing the use of antimicrobials and chemicals in marine environment, we decided to characterized local isolates of bacterium *T. maritimum*, choose the appropriate strain, produce autogenous vaccine and test its safety and efficacy in laboratory and farm conditions.

2) Methodology

2.1. Selection and characterisation of T. maritimum isolates and vaccine production

Primarily, we confirmed their affiliation by determination of the biochemical properties (using API 20E testing kits), and using molecular tools (testing for 16SrRNA, sequencing of this conserved gene and comparison of sequences with those deposited in GenBank) (Avendano-Herrera et al., 2017). Chosen isolates were tested for virulence by whole genome sequencing and the appropriate isolate was chosen for vaccine production. Whole genome sequencing (Perez-Pasqual et al., 2017) was performed using subcontractor MicrobesNG (UK) and showed homogeneity among isolates which was demonstrated by phylogenetic analysis and comparison with other isolates (Fig 6).

Chosen isolates were cultivated in Marine broth to obtain a starter culture which was delivered to subcontractor BIOCentar, Zagreb who produced required quantities of vaccine for testing in laboratory and field conditions.
The starter bacteria were fermented in bioreactor for production of formalin-inactivated whole-cell vaccine (FMC) with the final density of 3.1 measured at 600 nm (OD_{600}) after ultrasound homogenization. The fermentation was carried in 2 L fermenters in Marine Broth at 24°C, pH 7.1 and pO_2 between 20-100% during 72 hours. Homology of bacterial culture was checked at different bacterial media and after termination of fermentation, the culture was inactivated using dilution of 30% formaldehyde with its final concentration of 0.4%. Since *T. maritimum* is tending to create very strong biofilm (Fig. 7), the biofilm was destroyed using ultrasound homogenisation during one hour. Sterility was tested and vaccine was ready to be used for experiments with fish.

prof. Marco GALEOTTI DVM, Dipl. E.C.V.P.
University of Udine
Department of Agricultural, Food, Environmental and Animal Sciences
Via Sondrio, 2, Udine, 33100, Italy
+39 0432-558594
marco.galeotti@uniud.it

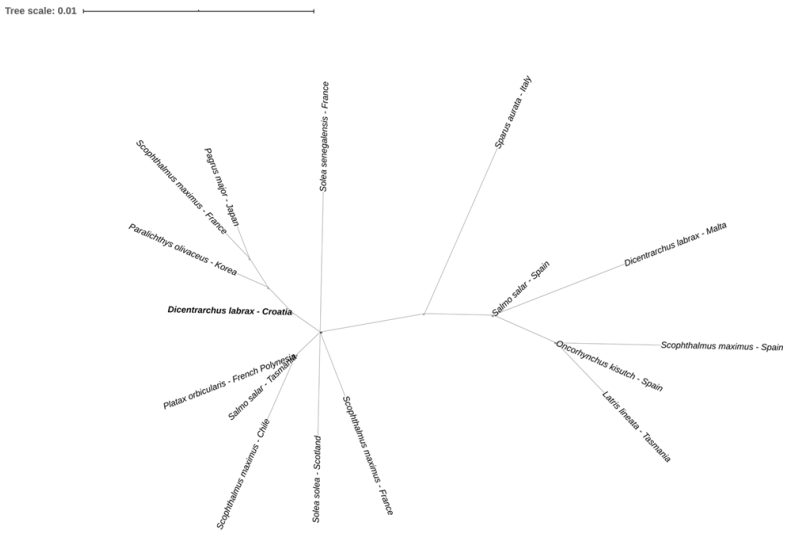

Figure 6. Phylogenetic analysis of Croatian selected isolates with isolates deposited in GenBank

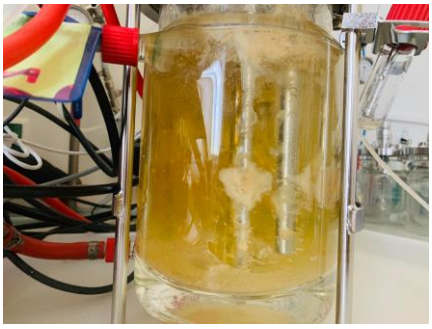

Figure 7. Biofilm formation of *T. maritimum* culture in bioreactor

3) Testing of safety and efficacy of vaccine in laboratory and farm conditions and obtained results

3.1. Laboratory experiments

The experimental vaccination was carried out in the premises of Institute of Oceanography and Fisheries (PP3) in Split. Experimental procedures involving animals in the study were conducted in accordance with the Laboratory Animal Management Principles of Croatia and all experimental protocols were approved by the Ethics Committee of the Institute of Oceanography and Fisheries (No. 134/2/2018).

Fish for experiments were tested for the presence of *T. maritimum* upon arrival to the experimental facility.

The experimental design consisted of 4 experimental groups (n=10) of seabass fry mean body weight of 10 grams in triplicates:

Group 1 – vaccination by immersion during 2 min in dilution of vaccine 1:10 to obtain 3.75 x 10^6 CFU mL^{-1}

Group 2 – vaccination by intraperitoneal injection (i/p) of 0.2 mL per fish (dose 6.0 x 10^7 mL^{-1})

Group 3 – mock vaccinated fish by immersion during 2 min in sea water

Group 4 – mock vaccinated fish by intraperitoneal injection of 0.1 mL PBS per fish

Before starting the experiment four groups consisting of 30 fish each were vaccinated by above-described protocols to test safety of vaccine and kept for period of 40 days. No mortality was recorded. Samples of gills, head kidney and spleen were collected from 6 specimen per group four, twenty-four hours and ten days after vaccination, preserved in RNAlater and frozen for testing of expression of genes of interest (Mitler et al., 2009). On the day 30, samples of blood were collected per each experimental group (n=6) and sera were frozen and delivered to LP for testing for the presence of specific IgM. PP1 produced bacterin which will be used in these analyses.

After vaccination fish were kept in their separated tanks, fed 1.5% body weight daily and checked for any changes and mortality. On 36th day fish were challenged following the scheme showed in the Table 1 where obtained results are alo summarized. The Relative Percentage of Survival was calculated based on the equation RPS= (1-($\frac{A}{B}$)) x100 where A represents the percentage of dead fish in experimental group and B represents percentage of dead fish in control group.

Table 1. Survival of fish vaccinated against T. maritimum after challenge with the same bacteria

Experimental group	Vaccination 09.06.2020.	Challenge 15.07.2020.	N° of fish per group	Mortality (N° of fish)	Survival rate (%)	RPS
4-8	IP 0.2 mL/fish (6.0 x 10^7 CFU/mL)	IP 0.2 mL/fish 7.2 x 10^7 CFU/mL	10	1	87	67,5
4-9			10	2		
4-10			10	1		
5-10	Mock i/p vaccinated control		30	12	60	
5-11	Bath– 2 min 100 ml vaccine + 900 mL seawater (final dilution 3.75 x 10^6 CFU/mL)	Bath 1.69 x 10^7 CFU/mL (150 mL 7.2 x 10^8 CFU/mL+350 mL seawater)	10	0	100	100
5-12			10	0		
5-13			10	0		
6-12	Mock bath vaccinated control		30	5	83	

The efficacy of vaccination was additionally evaluated by testing expression of gene of interest using real-time PCR. We selected genes coding for humoral and acquired immunity (IgT, IgM and p35) but also genes coding for cellular, innate immunity (IL1b and IL-10). The results obtained in material from gills, head kidney and spleen showed varying results shown in Fig. 8.

Results for expression of genes coding IgM failed although we used two sets of primers recommended by different authors (Miccoli et al., 2019; Piazzon et al., 2016; Hoare et al., 2017) while results for IL1b were inconsistent. Results for genes coding for humoral immunity proved that vaccination boost humoral immunity showing significant upregulation ten days after vaccination (IgT and p35) while in the case of IL-10 proinflammatory cytokine it is obvious that manipulation with fish is a strong activator of the immune response and they are upregulation four or 24 hours after manipulation and downregulated after ten days.

prof. Marco GALEOTTI DVM, Dipl. E.C.V.P.
University of Udine
Department of Agricultural, Food, Environmental and Animal Sciences
Via Sondrio, 2, Udine, 33100, Italy
+39 0432-558594
marco.galeotti@uniud.it

Sera were collected in laboratory vaccinated fish 30 days post vaccination and tested for specific IgM against *T. maritimum* bacterin using ELISA. Results showed that titre of specific IgM detected in immersion vaccinated fish was

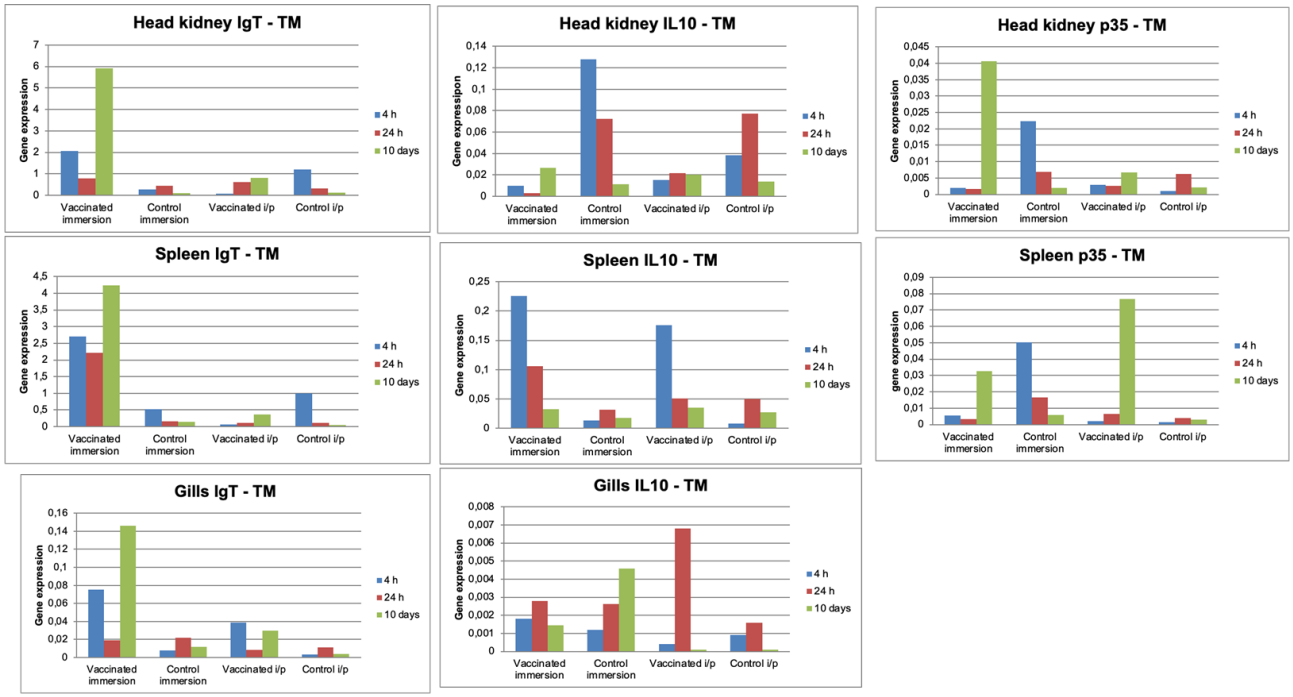

Figure 8. Results of gene expression after vaccination against *T. maritimum*

the same as in mock vaccinated fish (Fig. 9a), while specific IgM in intraperitoneally vaccinated fish showed significantly higher titer compared to fish vaccinated by i/p injection of PBS.

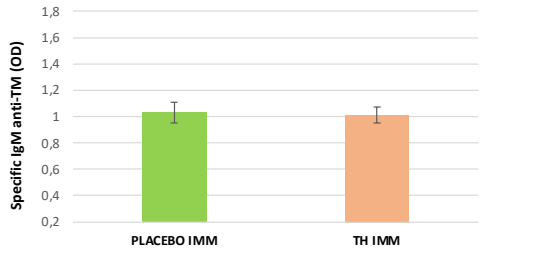

Figure 9a. Immersion vaccination

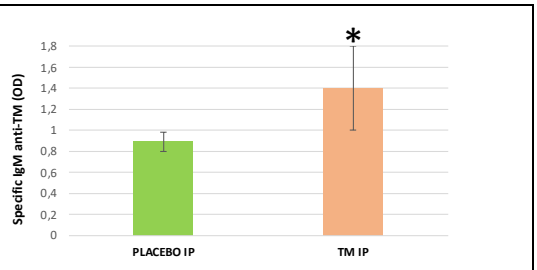

Figure 9b. Intraperitoneal vaccination

3.2 Farm testing of vaccine efficacy

Four experimental groups were placed in small cages (4m x 4m x 4m) established on the Friškina Fish Farm (PP8). In each cage sea bass fry (n=1250) mean weight of 13,18 grams was placed. Each group was vaccinated as follows:

i) vaccination against *Vibrio harveyi* by immersion in dilution (3.3 x10^8 CFU ml-^{1}) 1:10 to obtain 10 L during 1 min

ii) vaccination against *T. maritimum* by immersion in dilution (7.4 x10^6 CFU ml-^{1}) 1:10 to obtain 10 L during 2 min

iii) Vaccination by immersion in mixure of both vaccine in dilution 1:10 during 2 minutes

prof. Marco GALEOTTI DVM, Dipl. E.C.V.P.
University of Udine
Department of Agricultural, Food, Environmental and Animal Sciences

Via Sondrio, 2, Udine, 33100, Italy
+39 0432-558594
marco.galeotti@uniud.it
www.italy-croatia.eu/adriaquanet

iv) Control, non-vaccinated group

All experimental groups showed high survival rate (95%) during the period of one year after vaccination and there were no outbreaks or positive findings neither of vibriosis caused by *Vibrio harveyi* nor tenacibaculosis caused by *T. maritimum.* Blood was collected from 6 specimen of each experimental group two and ten months after vaccination and sera were tested for specific anti-TM IgM or specific anti-VH IgM and also for total IgM. The obtained results showed that specific anti-TM IgM were higher in the sera of specimen vaccinated both by vaccine against *T. maritimum* and also with mixture of both vaccines compared to the control group (Fig. 10a & b) with higher concentration after two months than after 10 months. Total IgM were also higher compared to control group two- and ten-months post vaccination (Fig. 11 a & b). However, the titre is even higher ten month after vaccination what could be explained by the fact that seabass was additionally immunized by the presence of the bacteria in the farm environment.

Two months after vaccination

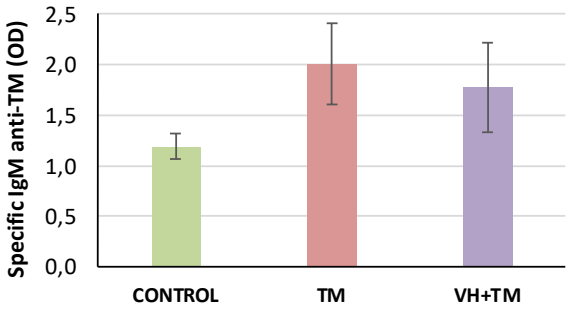

Figure 10a. Specific IgM anti-TM in sera of seabass

Ten months after vaccination

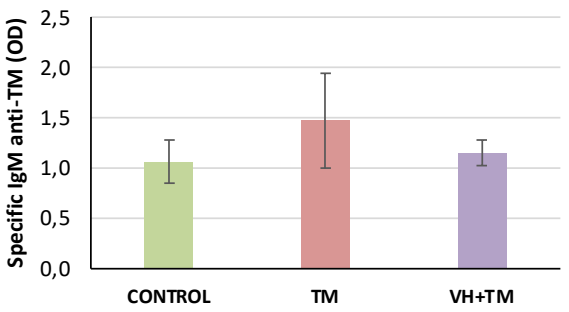

Figure 10b. Specific IgM anti-TM in sera of sea bass

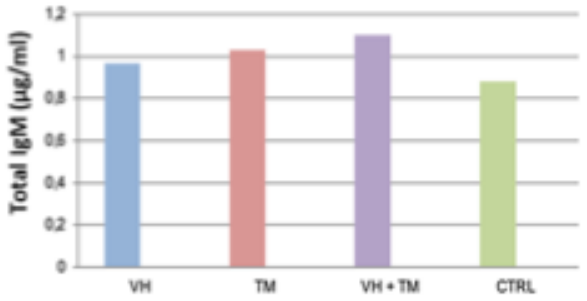

Figure 11a. Total IgM in sera if seabass

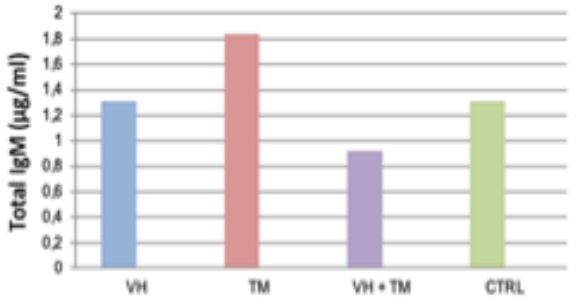

Figure 11b. Total IgM in sera of seabass

4) Conclusions

Laboratory trials showed that produced autologous vaccine against T. maritimum was safe and have promising efficacy during both way of application and challenge. Probably, more confident results of RPS would be obtained if fish vaccinated by both way of application were challenged both by bath and intraperitoneal injection of the bacterial. These initial results were supported by gene expression measured after vaccination, same as by higher titer of anti-TM

prof. Marco GALEOTTI DVM, Dipl. E.C.V.P.
University of Udine
Department of Agricultural, Food, Environmental and Animal Sciences
Via Sondrio, 2, Udine, 33100, Italy
+39 0432-558594
marco.galeotti@uniud.it

IgM in sera of vaccinated fish thirty days post vaccination. Conclusively, obtained results are giving a very good basis for further research as there is a room to improve vaccine by addition of some adjuvants or other immunostimulants and how to improve the efficacy testing protocols. Farm trials did not give clear results but it is obvious that specific IgM were higher indicating acquired immunity stimulation compared to unvaccinated fish while it seems that measuring of total IgM was not appropriate methodology for evaluation of the vaccine efficacy.

5) Annexes are not available.

6) List of references

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prof. Marco GALEOTTI DVM, Dipl. E.C.V.P.
University of Udine
Department of Agricultural, Food, Environmental and Animal Sciences

Via Sondrio, 2, Udine, 33100, Italy
+39 0432-558594
marco.galeotti@uniud.it

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7) List of equipment

Ref.	Image (photo with the project label on)	Description (name of the equipment and short description of the object)	Station Town and PP where is places
1		**Conventional PCR thermocycler** is used for molecular diagnostics of bacteria used for vaccine preparation and also for health checks during the experiments	Croatian Veterinary Institute, Laboratory for Fish Pathology, Zagreb, 10000
2		**PCR cabinet** Used for preparation of the components needed for the preparation of master mix (mixture for performing both conventional and real-time PCR)	Croatian Veterinary Institute, Laboratory for Fish Pathology, Zagreb, 10000
3		**Thermoshaker** is used as an incubator and a constant orbital mixer in one machine for samples that need to be kept at a specific temperature and continuously mixed. Used to produce uniform samples, provide an ideal growth environment, and facilitate DNA amplification	Croatian Veterinary Institute, Laboratory for Fish Pathology, Zagreb, 10000

4		**Minicentrifuge** Used for centrifugation of small quantities of ingredients used in molecular techniques	Croatian Veterinary Institute, Laboratory for Fish Pathology, Zagreb, 10000
5		**Centrifuge with vortex** Used for centrifugation and homogenisation of samples and ingredients used for molecular diagnostic techniques	Croatian Veterinary Institute, Laboratory for Fish Pathology, Zagreb, 10000
6		**Real-time PCR thermocycler** Used for real-time testing of bacteria and also for testing of the genes of interest expressed after vaccination carried out as a means of vaccination efficiency evaluation.	Croatian Veterinary Institute, Laboratory for Fish Pathology, Zagreb, 10000
7		**Speed mill** for homogenisation of tissue samples before isolation of nucleic acid	Croatian Veterinary Institute, Laboratory for Fish Pathology, Zagreb, 10000
8		Station for **automated isolation** of nucleic acid for diagnostics of bacterial diseases but also for isolation of RNA for testing for gene of interest expression	Croatian Veterinary Institute, Laboratory for Fish Pathology, Zagreb, 10000

- **PART 2**

A. CONTRIBUTION TO EUSAIR

Please provide a description of the project contribution to the EUSAIR in terms of synergy with the Strategy's pillars and alignment of implemented project's activities with the Action Plans and labelled projects.

Project contributes to the EUSAIR Strategy's pillar "blue growth" and through innovation and development of the sustainability of aquaculture in the Adriatic Sea establishes a basis for the development of aquaculture in the whole EUSAIR region. In particular, a network of academia and industry worked together in the enhancing profitable, high-quality and sustainable aquaculture production which is capable to contributes to job creation and economical growth of rural and outlying island communities as well as to supply of healthy food products, respecting the EU and international rules. The results of task 4.1 will decrease the economic burden of diseases to the marine aquaculture sector and can be easily transferred to other territories of the EUSAIR especially those missing specialised research centres as well as other Mediterranean areas.

B. CONTRIBUTION TO HORIZONTAL PRINCIPLES

Please provide a description of the project contribution to the horizontal principles of equality between men and women, non-discrimination and sustainable development.

The project gathered different experts based on the skills regardless of race, nationality, ethnic origin, religion, disability, age or sexual orientation. Two research assistants were employed based on the principle of quality and fair treatment, access to training, promotion. The focus was on the one common goal, or rather on the promotion of a healthy and sustainable product from the Adriatic regions, bringing together farmers, scientists, consumers, veterinarians and experts in the field. In particular, task 4.1. contributes sustainable aquaculture and sustainable use of marine environment for safe, high quality food production as vaccination directly results in: 1) antimicrobials use avoidance and thus protects health of farmed fish, environment and consumers; 2) better feed digestion and again protection of environment from feed waste; 3) finally, better productivity in aquaculture sector and welfare for the society in whole

C. COMMUNICATION ACTIVITIES

Please refer to the Final Communication Report template and provide a summary on the main achievements trying also to identify which were the most successful communication tools in reaching general public/decision makers/other target groups.

All project activities were disseminated through different channels such as PP1 webpage, Facebook portal, Veterina portal and through different virtual and face to face conferences. Many of experts were reached through virtual and online workshop organised to disseminate the project results. However, the most important events were press conference organised in PP1 premises in Rijeka where PP1 and PP5 presented the project outputs as well as the press conference immediately before the final conference in Zadar on 3rd June 2022 which hosted decision makers (Ministry of agriculture, Fisheries directorate). These press conferences raised a huge interest of journalists and reached huge number of general public. Moreover, partners were invited to several TV and radio show on public TV and radio where project goals and results were disseminated.

prof. Marco GALEOTTI DVM, Dipl. E.C.V.P.
University of Udine
Department of Agricultural, Food, Environmental and Animal Sciences
Via Sondrio, 2, Udine, 33100, Italy
+39 0432-558594
marco.galeotti@uniud.it
www.italy-croatia.eu/adriaquanet

D. **NATURA 2000**

Please describe, if it is the case, measures foreseen and implemented by the project:

a) In case the project involved Natura 2000 sites, describe what measure the project envisaged and implemented to avoid any negative impact: Not applicable
b) In case the project had a positive effect on Natura 2000 sites, please describe which measure the project has foreseen and implemented in order to reach a direct or indirect positive impact: Not applicable

E. TYPES OF ACTIONS ADDRESSED (as defined in the Cooperation Programme)

These are our primary objective's types of actions, that we addressed by the Project:

Specific Objectives	***Types of action***	***the most relevant one within the SO addressed by your project***
1.1 Enhance the framework conditions for innovation in the relevant sectors of the blue economy within the cooperation area	*Joint projects and actions aimed at creating platforms, networks and at supporting exchange of good practices in order to enhance the knowledge transfer and capitalization of achieved results in the field of blue economy*	***X***
	Actions aimed at cluster cooperation, joint pilot initiatives in order to boost the creation of marketable innovative processes and products, in the field of blue economy	***X***

F. TYPES OF OUTPUTS PRODUCED

Specify the types of outputs generated by your activity that are reported here and provide a brief description

prof. Marco GALEOTTI DVM, Dipl. E.C.V.P.
University of Udine
Department of Agricultural, Food, Environmental and Animal Sciences
Via Sondrio, 2, Udine, 33100, Italy
+39 0432-558594
marco.galeotti@uniud.it
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Output typology	Description
Trainings	There were 3 online training and 3 face to face training in Croatia and Italy with participation of fish health experts and fish farmers with demonstration of safety, non-toxicity and results of laboratory and farming testing of efficacy of autogenous vaccine against bacterium *Tenacibaculum maritimum*.
Monitoring systems	Monitoring system of efficacy control was established on the farm with experimental vaccination during the project duration.
SMEs clusters	Klaster marikultura, PP7 was a very important SMEs cluster, a partner in the project who presented the link between scientific and industrial partners in the AdriAquaNet project. All project outputs printed material was delivered personally during the physical workshop or by mail during the lock-down to members of the Klaster marikultura. Moreover, PP1, PP3 and subcontractor I-riba are also members of the Klaster marikultura.
New networks	PP1 strenghtened the relationship with Croatian partners and established additionally very strong and high quality cooperation with Italian partner and some new idea were arouse how to deepen cooperation in the Blue growth field but also how to spread the cooperation in the field of food quality and safety. However, some new relationships were established between Croatian scientists and Italian fish farmers and vice versa, Italian scientists and Croatian fish farmets. Besides testing the vaccine against T. maritimum in Croatian farm, some quantites were provided to be tested in Italian hatcheries which are exporting some quantities of fry to Croatia.
Platforms	
Adaptation plan	
Building renovation	
Others (please specify)	

prof. Marco GALEOTTI DVM, Dipl. E.C.V.P.
University of Udine
Department of Agricultural, Food, Environmental and Animal Sciences
Via Sondrio, 2, Udine, 33100, Italy
+39 0432-558594
marco.galeotti@uniud.it

G. TYPOLOGY OF IMPACTS

Please indicate what type of impact(s) your project has had. You can choose more than one answer. For each tangible impact selected, please provide a concrete example from your project, where possible supported by quantitative information.

TANGIBLE IMPACTS

Tangible impacts	Example/ quantitative information
Improved access to services	National production of autogenous vaccine will simplity and facilitate access to the preventive measures for Croatian farmers. A handbook on the Vaccination Strategies in Hatcheries and Fish Farms is giving them tutorial on how to manage the most important bacterial fish diseases in Adriatic marine aquaculture.
Cost savings	Bacterial diseases are causing high economic burden on the profitability of marine aquaculture. The cycle from observing the first symptom of the disease, notifying the fish health expert, diagnostic analysis with antibiotic susceptibility testing is time consuming, ordering medicinal feed is requiring few more days and increase the cost of bacterial diseases management. Additional work efforts should be engaged in disinfection, dead fish collection and storage to avoid the disease spreading are also time and money consuming. All these expenses mentioned could be avoided by timely vaccination and the productivity of the farming is improving.
Time savings	As it was described in the previous paragraph, farm workers are not engaged in dead fish collection or preparation of medicinal feed and they have more time to dedicate to the hygiene (net changing and reparing, observing the fish feeding, etc.) and quality of fish management.
Reduced energy consumption	NA
Reduced environmental impact	Antibacterial treatment is posing a risk not only for fish consumers in the case of accumulation of residues in the fish flesch or development of antimicrobial resistance but also the risk for the environment. Genes of antimicrobial resistance are spread to autochtonous bacteria present in marine environment and could be transferred to other organisms or humans. Therefore vaccination is mitigating the risk of imprudent and unreasonable use of antimicrobials in marine aquaculture.
(Man-made, natural) risk reduction	All interventions including diseases treatment in the aquatic environment have impact on this environment. Farming of fish in cages have impact on the marine environment and it should be as much as it is possible sustainable. Diseases prevention by vaccination is mitigating the environmental risk in two ways: 1) avoid introduction of medicines in the environment and concomitant prevention of deleterious impact of antimicrobials on the living organisms and humans; 2) prevention of harmful diseases spreading to the susceptible marine organisms living in vicinity of fish farms
Business development	Better survival and profitability of seabass and seabream farming will enable new investment and increasing of production either on the same site if it is

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University of Udine
Department of Agricultural, Food, Environmental and Animal Sciences
Via Sondrio, 2, Udine, 33100, Italy
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marco.galeotti@uniud.it
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	available based on the carrying capacity of the farming site or by inclusion of the new site for farming.
Job creation	New investment and new skills acquired due to the outputs of the AdriAquaNet project will create new job during the whole year in rural and outlying island communities and make a step forward from possibility of employment only during the summer touristic season.
Improved competitiveness	Adriatic marine aquculture will offer more sustainable farmed fish on the mutual EU market.
Other tangible impacts (specify)	Marine aquaculture will become more resilient and modern industry promoting sustainability in the fish health management.

INTANGIBLE IMPACTS

Intangible impacts	**Example/quantitative information**
Building institutional capacity	PP1 build capacity for molecular diagnostic tools, acquired skills to produce, experimentally test and evaluate efficacy of vaccination using molecular tools and grew in applied scientific excellence.
Raising awareness	Communication activities presenting a new approach in diseases management improve knowledge of consumers on the safety of Adriatic marine aquaculture.
Changing attitudes and behaviour	Positive experience with vaccination using autogenous vaccines will help in moving forward to prevention rather than cure fish during farming.
Influencing policies	Strategy of the aquaculture development is promoting marine aquaculture efficiency, competitiveness and resilience.
Improving social cohesion	Contribution to the development of the marine aquaculture will contribute to the development of rural and outlying island communities and enable family life in these area with high emigration to the coomunities where young people could find employment during the whole years.
Leveraging synergies	

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