AdriAquaNet

Enhancing Innovation and Sustainability in Adriatic Aquaculture

Deliverable WP4 task 4.2.2

Technical-scientific report of the fish microbiome, probiotic candidates and the effect of feeding trials on the fish growth and immunity

PP3 IOF - Split, 30.06.2022

European Regional Development Fund

www.italy-croatia.eu/adriaquanet
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Project Acronym:	**AdriAquaNet**
Project Title:	**Enhancing Innovation and Sustainability in Adriatic Aquaculture**
Start of the project:	**01/01/2019**
Duration:	**42 months**
WP/activity:	**WP4, Activity 4.2.2**
Deliverable name:	**Technical-scientific report of the fish microbiome, probiotic candidates and the effect of feeding trials on the fish growth and immunity**
WP leader:	**UNIUD**
Author (s):	**J. Hribar in collaboration with LP, PP1, PP2, PP4, PP8 and PP10**
Delivery date:	**30/06/2022**
Status:	**Final**

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- **PART 1**

  The first part provides details and assessment of the WP objectives related to the primary subject of your report and the implementation and results, compared to the information already provided in the different progress reports, in order to give back a cumulative illustration of what the project delivered in relation to this task;

- **PART 2**

  The second part provides the final results and a collection of data from the WP and project in relation to the General objectives at the Programme level that we will need to add to the final report.

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

PART 1

A. REPORT HIGHLIGHTS

Please provide a cumulative short overview of the project WP task 's achievements

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Marine fish are accepted as a significant source of nutritionally valuable compounds such as unsaturated fatty acids. Increasing dietary trends and changes in dietary habits in the last few decades have increased the demand for high-quality fisheries products. Traditional catch-based fisheries cannot supply the market with enough fish, making aquaculture alternative to traditional fisheries. However, in an era of increasing antimicrobial resistance to already a poor selection of chemotherapeutics used in aquaculture systems it is imperative to find alternatives to use of antibiotics which could be used as prophylactic measures rather than curative ones. One such approach is the use of probiotics, whose beneficial effects on fish growth and overall health status have been proven on many occasions.

For these reasons one of the objectives of the AdriAquaNet project was to identify autochthonous intestinal bacteria of cage reared fish to potentially be used as probiotics supplemented in the fish feed and tested in feeding trial on a farm (PP8 – Friškina Ltd.).

Institute of Oceanography and Fisheries (IOF) – Project Partner 3 (PP3) sampled sea bass and sea bream from Croatian farms to get insight into the diversity of cultured fish intestinal microbiota as well as to identify culturable bacterial isolates from which a potentially probiotic strain could be selected. After identification and characterization of several isolates, a single Bacillus *spp. isolate was selected as a probiotic candidate. The isolate was tested* in vitro *for its ability to stimulate fish immune cells. Afterwards, large quantities of the bacteria were prepared and incorporated in feed containing 10% black soldier fly (*Hermetia illucens*) protein (commercially done by* BIOCenter and Naturalleva, respectively). *During the six-month farm feeding trial fish was fed novel diet formulation with (group 1) or without the probiotic supplementation (group 2) as well as commercial diet (group 3). Biometric parameters were regularly checked to assess the growth performance. At predefined time points, samples of either whole or proximal intestine were taken to assess the overall intestinal health status and detect possible changes caused by the novel feed formulations (composition of microbial communities, histo-morphology, gene expression).*

Both group 1 and group 2 performed well in terms of growth. The intestinal health status of both groups was not compromised due to the change in feed, more specifically no inflammatory or degenerative changes were noticed. Addition of the insect protein alone was beneficial for intestinal health status and this effect was further augmented by the probiotic supplementation. Several inflammatory genes were significantly expressed in group 1, but their expression was reversed by the probiotic supplementation. Taken together, the results of this task strongly confirm the beneficial effects of selected probiotic strain on maintaining intestinal homeostasis.

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
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marco.galeotti@uniud.it

B. WP PROJECT OUTPUTS AND RESULTS

Specific 2: Promote fish health and provide "healthy and safe" fish to consumers

Summary and the presentation of the activities and the results:

During June and July 2019 PP3 collected sea bream and sea bass from eight farms along the Croatian Adriatic coast to assess the diversity of Adriatic cage reared fish intestinal microbiota and identify culturable isolates (N=150) from which a potential probiotic strain could be selected. Based on literature search and biochemical parameters a single *Bacillus* spp. isolate was selected as a potential probiotic to be tested in a farm feeding trial. The isolate was first tested on sea bream and sea bass peripheral blood leukocytes in an *in vitro* stimulation assay and expression analysis of several immune related genes was done. Afterwards, large quantities of the isolate were cultured by subcontractor Biocentre (Zagreb, Croatia) and shipped to Naturalleva (Cologna Veneta, Italy), a subcontractor who produced fish feed to be tested on a farm. Three different feeds were prepared: i) commercial (control), ii) feed with the addition of 10% crude protein from black soldier fly (*Hermetia illucens*), iii) feed with insect protein supplemented with *Bacillus* spp.

Cages with experimental fish at Friškina Ltd. farm

A six-month farm feeding trial starting end of May 2020. was carried out in Friškina Ltd. farm near Rogoznica. Three groups (in duplicate cages) of juvenile sea bream (initial body weight ~121 g) were assigned to each of the experimental feeds. The group receiving the feed supplemented with insect protein and probiotic was fed according to a switch-on/switch-off strategy, meaning that the fish was

prof. Marco GALEOTTI DVM, Dipl. E.C.V.P.
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Via Sondrio, 2, Udine, 33100, Italy
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marco.galeotti@uniud.it

fed for two weeks with this diet followed by two weeks of feeding with insect protein supplemented diet. Such strategy was followed for the whole feeding trial.

Sampling at Friškina Ltd. farm

Two weeks, one month and six months after the start of the feeding trial six fish per feeding group were sacrificed and samples taken for proximal intestine histomorphology, proximal intestine gene expression analysis using customised qPCR arrays (subcontractor Biotechvana, Spain) with markers for immune response, epithelial integrity, nutrient transport and mucus production and whole intestine microbiota analysis (subcontractor MrDNA Lab, USA). Additionally, one month after the start of the feeding trial and at the end of trial, blood was taken from six fish per group for serum biochemical parameters analysis. All these parameters are indicative of fish intestinal health and would point out to any possible changes due to the application of insect protein and/or selected probiotic strain. Besides considering the parameters of the immune status and intestinal health, biometric parameters were regularly checked to assess fish growth performance due to diet composition.

Histomorphological analysis of proximal intestine showed no inflammatory or degenerative changes in any of the experimental fish groups. Only an increase in mucus producing goblet cells was evident in fish fed probiotic supplemented diet, indicating a beneficial effect of probiotic supplementation on maintaining intestinal mucus barrier.

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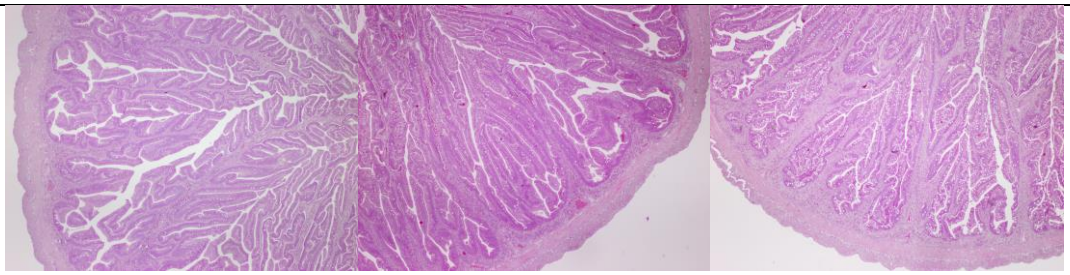

Histological appearance of proximal intestine of fish fed i) insect protein supplemented diet, ii) insect protein and probiotic supplemented diet, iii) control diet

A statistically significant effect of sampling time was found for 26 out of 44 selected genes. Dietary treatment significantly affected 19 genes, while interaction of time and diet was significant for 14 genes. Multivariate analysis of dietary treatments did not discriminate between the three diets mostly due to the overlapping of the two insect-based diets. In total, 17 genes showed higher contribution to the variation between the diets and on heatmap clustering grouped the two insect-based diets apart from the control diet. Many markers of epithelia integrity (e. g. claudins and cadherin) were slightly up-regulated in the fish fed insect protein supplemented diet, and probiotic supplementation specifically amplified their up-regulation. Several of the inflammatory and immune-regulatory genes reached maximum expression in fish fed insect protein supplemented diet, and probiotic supplementation reversed their expression to the values close to those of the control group.

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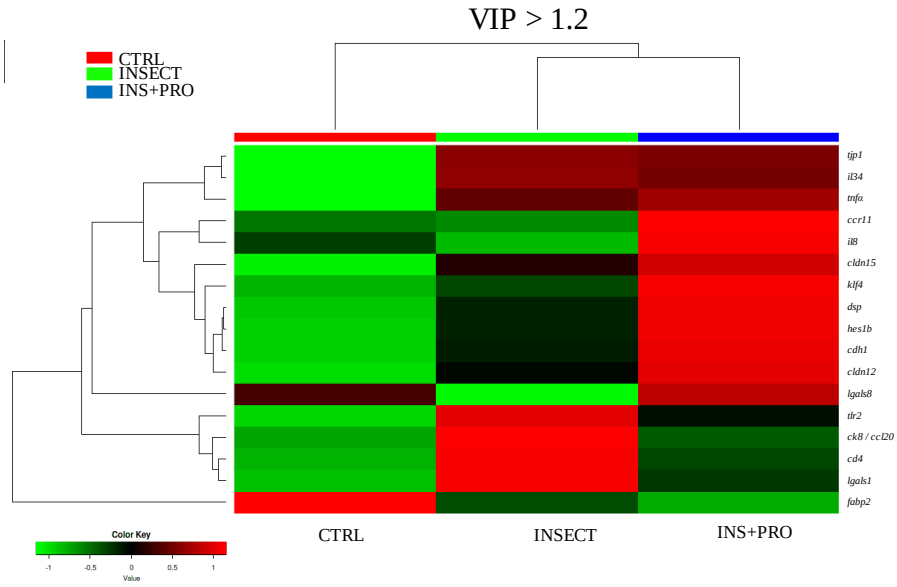

Clustering heatmap of the effects of dietary treatment on the gene expression of selected genes in proximal intestine. Colours represent relative fold-change variations (green, down-regulation; red, up-regulation)

Results of the microbiota analysis showed significant differences between at least two groups for 27 genera with mean relative abundance of 0.1% or higher. Noteworthy is that five of these genera belong to the group of lactic acid bacteria which have known beneficial effect on intestinal homeostasis. The highest number of significant pairwise differences was found for the genus *Paenibacillus*, whose members produce various enzymes, such polysaccharide-degrading enzymes and proteases, and antimicrobial substances that can affect a wide-spectrum of microorganisms. However, the influence of environmental conditions on the composition of the intestinal microbial communities cannot be ruled out.

Regarding the serum biochemical parameters, no significant changes were detected for any of the markers, except for the antiprotease activity in fish fed probiotic-supplemented diet one month after the start of the feeding trial.

Reasons of discrepancies between planned and realized outputs (if any)

We had no discrepancies between the planned and realized outputs.

Impact of outputs underachievement on project results

Not applicable.

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Additional results (was the project able to reach additional outputs /results besides those foreseen in AF? Not applicable.

C. DURABILITY AND TRANSFERABILITY OF THE PROJECT AND ITS RESULTS

Please describe shortly:

How will the outputs and results be maintained and developed further after project end? Results of the farm feeding trial demonstrated beneficial effect of autochthonous *Bacillus* supplementation on intestinal health and homeostasis maintenance of cultured sea bream. These outputs could be further developed by pinpointing those factors of the isolate responsible for its beneficial effect, potentially avoiding the use of whole bacterial cells and simplifying feed preparation and its storage. Moreover, different feeding strategies than the one applied during this project should also be tested to exploit the probiotic potential of the selected strain to the fullest. Finally, a challenge with a pathogenic strain following the probiotic application would give a final confirmation whether the selected *Bacillus* isolate could truly serve as a novel probiotic in sea bream aquaculture and as a prophylactic measure in preventing diseases and promoting fish growth and health.
How has the availability of project results and outputs for general public and other stakeholders been ensured during the project life and eventually after the project end? During the project several online (during the COVID-19 pandemic) and on-site trainings and workshops were organised for the stakeholders and public interested in the topic, where the results of the specific task were presented. Furthermore, two scientific articles are currently being prepared for publishing in peer reviewed journals after the project termination where the results of the task will be presented discussed and new research perspectives proposed.

D. CAPITALISATION OF RESULTS

Please provide information about capitalisation:

Was the project able to capitalise or influence future calls or other projects? Please specify main results or output to be considered for future capitalisation action. **Are there any obstacles of legal or administrative nature that the project has encountered and which hampered cooperation? Is there any room to solve these obstacles?** The results of this project will be useful for future calls and other projects to explore different feeding strategies to exploit the probiotic potential of the selected strain to the fullest. Furthermore, the results and outputs of the project could be elevated by performing challenge with pathogenic strain(s) to affirm whether the selected probiotic strain could also serve as a good prophylactic measure in disease prevention not just maintaining the intestinal health status and homeostasis. No obstacles have been encountered during the project implementation.

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marco.galeotti@uniud.it

E. PARTNERSHIP COOPERATION

Please provide an assessment of the participation and involvement of the partners in the project, answering the following questions:

Which Partners were active in your WP and the activities related to the report? Were all the Partners involved also active? Besides PP8 (Friškina Ltd.) at whose farm the feeding trial was conducted, LP (University of Udine), PP1 (Croatian Veterinary Institute), PP2 (University of Trieste) and PP4 (Istituto Zooprofilattico Sperimentale delle Venezie) were also involved in WP activities. The cooperation between the partners has been achieved in full sense as all the partners contributed to the WP within their area of expertise. Additionally, three companies (Biocentre, Naturalleva and Biotechvana) and one public University (University of Osijek) were also involved as subcontractors of PP3.
Were they all able to attract other local/regional actors and involve them in the project activities? No as the PP3 subcontracted three companies and one public University to cover several of the activities of the WP.
What was the added value given by the cooperation? With the cooperation with the three subcontracting companies, we were able to ensure undisturbed feed supply during the farm feeding trial (Biocentre, Naturalleva) and get a more detailed insight into the effect of the probiotic on the intestinal health of experimentally fed fish.
Which were the main problems encountered? The main problem encountered was the lack of sufficient number of replicates per experimental group as increasing the number of cages would have significantly interfered with normal production process at Friškina farm.
Was the project able to create links with other projects? Not applicable.
Will the PPs cooperate in the future even without funding (if yes explain the main aims of this cooperation)? Yes, but mainly in trainings organisation an results dissemination.

F. TARGET GROUPS INVOLVEMENT

Please list the main target groups that benefited from your WP project's achievements as inserted in the relevant Report Section in SIU that you will find on the left (the numbers are our project numbers). In few word provide further details on how they were able to make use of the outputs/ results of the project.

TARGET GROUPS	***Description***
SMEs (50)	*1 farm directly involved in the feeding trial.*
Universities, technology transfer institutions, research institutions (10)	*LP, PP1, PP2, PP3, PP4, University of Osijek*
NGOs, associations, innovation agencies, business incubators,	

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cluster management bodies and networks (5)	
Centers of R excellence (5)	
Local, regional and national public authorities (10)	
General public (1000)	*Dissemination of the results on PP3 online channels (website, social media), on the AdriAquaNet project website and as a published scientific article in a high impact journal.*

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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.

The AdriAquaNet project contributes to the EUSAIR strategy within the Pillar 1 "Blue growth", Topic 2 "Fisheries and aquaculture", aiming at enhancing the Adriatic area aquaculture by developing a sector that is both economically and environmentally sustainable. The activities within the task 4.2.2. are important in terms of environmental and economic sustainability, as novel probiotic tested within the project could serve as a good prophylactic measure for disease prevention and reduction of production costs and environmental impact of aquaculture through the use of chemotherapeutics. The project results and outputs can easily be transferred to other territories of the EUSAIR region.

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 focus of the project was development and promotion of a healthy and sustainable aquaculture product from the Adriatic region and bringing together different interested parties (scientists, farmers, consumers, veterinarians…). To achieve that, staff participating in the project was engaged based on their personal characteristics, complying with the equal opportunities and without any discrimination based on gender, race, nationality, ethnic origin, religion, disability, age or sexual orientation.

A social component of the project should also be accentuated as increased sustainability and reduced production costs will help ensure continuous production and higher profitability for the farmers, providing at the same time permanent employment opportunities.

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.

Activities within task 4.2 performed to produce the present deliverable, in particular field work at the Friškina Ltd. farm and dissemination of the results have been documented with photos and videos by PP3 staff and PP2 subcontractor.

Task 4.2 main activities and results have been presented to a broad audience of scientists and specialists dealing with fish health on the EAFP 2021 conference in form of an oral presentation. The project and part of the results of the present task have also been presented as part of a conference workshop "How outputs from EU projects can upgrade health management in Mediterranean aquaculture". Furthermore, the activities and main results have been communicated with different stakeholders (farmers, veterinarians, local authorities…) during one online (February 24th, 2022) and one on-site training held in Zadar (June 2nd, 2022), with both trainings reaching high number of attendees. Finally, summary of the main activities and most important results has also been presented at two final conferences in Zadar (June 3rd, 2022) and Udine (June 20th, 2022), respectively.

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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.

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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

Output typology	**Description**
Trainings	2 training sessions (24.02, 02.06.2022) have been performed (online on-site) where the feeding strategy and the effect of probiotics supplementation have presented to stakeholders.
Monitoring systems	Not applicable.
SMEs clusters	Not applicable.
New networks	Not applicable.
Platforms	Not applicable.
Adaptation plan	Not applicable.
Building renovation	Not applicable.
Others (please specify)	Not applicable.

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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	/
Cost savings	Lower production expenses during the project duration due to purchasing of novel feeds from the project budget.
Time savings	/
Reduced energy consumption	/
Reduced environmental impact	Reduced environmental impact through improved immune status and reduced need of using chemotherapeutics.
(Man-made, natural) risk reduction	/
Business development	/
Job creation	/
Improved competitiveness	/
Other tangible impacts (specify)	/

INTANGIBLE IMPACTS

Intangible impacts	Example/quantitative information
Building institutional capacity	
Raising awareness	
Changing attitudes and	The results of the present task clearly showed the beneficial effects of

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

behavior	probiotic supplementation on fish gut health. Adopting the supplementation of fish feeds with probiotic isolates in the production process will help prevent the diseases outbreaks (at least some), reduced the need to use chemotherapeutics and in turn reduce the environmental impact of the aquaculture.
Influencing policies	
Improving social cohesion	
Leveraging synergies	
Other intangible impacts (Specify)	Final consumers are being increasingly aware of the environmental impact of aquaculture and are becoming more prone to consuming fish that has been cultured without the use of chemotherapeutics and with reduced environmental impact.

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