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Enhancing Innovation and Sustainability in the Adriatic Aquaculture

VACCINATION STRATEGIES IN ADRIATIC HATCHERIES AND FISH FARMS

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FOREWORD

AdriAquaNet (Enhancing Innovation and Sustainability in Adriatic Aquaculture) is an Interreg Italy-Croatia V-A 2014-2020 project which falls into Priority Axis 1 “Blue innovation – Enhance the framework conditions for innovation in the relevant sectors of the blue economy within the cooperation area”.

The main goal of the project is to strengthen sustainable aquaculture in the Adriatic Sea by transferring the advanced knowledge and new technologies through the whole aquaculture supply chain, from the management of the production on the farm to the market of the processed product. The project is conceived to intervene in three aspects of the value chain:

1. Improvement of the farming procedure through innovative feed formula and feeding procedure to improve the quality of fish and to save the environment and at the same time to implement the technology for energy saving.
2. Implementing a new approach to the health and welfare management through vaccination against bacterial diseases and application of natural products for treatments
3. Developing the guidelines for fish consumers by assessing the fish safety and quality, sensory and nutritional properties and health benefits and eventually, present all these facts through a comprehensive marketing campaign to consumers of the Adriatic region

Manual on “Vaccination Strategies in Adriatic Hatcheries and Fish Farms” is a document intended for European seabass and gilthead seabream farmers, veterinarians involved in Adriatic marine aquaculture health management, representatives of the pharmaceutical industry and all other stakeholders dealing with fish health management. The document provides basic information on the immune system of both fish species allowing readers to understand the importance of immunoprophylaxis in fish

farming and also presents a review of the economically most important bacterial diseases affecting mentioned species and practical advice needed during the vaccination implementation. Thus, activities and research realised within the work package 4 “R&I to improve health and sustainability in aquaculture”, activity 4.1. Vaccine production and vaccination strategy are explained and described here.

Authors are partners in the AdriAquaNet, leading experts in the field of fish health management from both sides of the Adriatic Sea, Italy and Croatia supported by experts from partner fish farms and stakeholder’s hatchery and they are quoted by alphabetical order:

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Although Cromaris is not a partner in the AdriAquaNet project we’d like to thank them for their very kind cooperation and readiness to support all research and activities of this regional project.

Snježana Zrnčić and Marco Galeotti

1. INTRODUCTION

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Farming of European sea bass (*Dicentrarchus labrax*) and gilthead sea bream (*Sparus aurata*) is a very important activity in the Adriatic region, both along the Italian and Croatian coasts. Currently, infectious diseases still represent a bottleneck in the development of the aquaculture industry (Fernandez Sanchez et al. 2021). Although the marine environment favours the survival of bacteria outside their host, there are only a few of them inhabiting the Mediterranean marine environment that are capable to provoke the disease (Pujalte et al. 2003). Among bacterial pathogens causing losses in farmed sea bass and sea bream, the most frequent, harmful and economically important are bacteria from the genus *Vibrio*, namely *Vibrio anguillarum* and *Photobacterium damselae* subsp. *piscicida*, and recently the emerging pathogen *Vibrio harveyi* (Mancuso 2014). Besides, *Tenacibaculum* spp. infections are considered among the most important diseases of sea bass (Zrnčić and Pavlinec 2020). However, a bacterial disease outbreak is not necessarily caused by a single pathogen but may involve a synergic interaction between different bacterial strains from two or more taxa.

A bacterial infection causing mass mortalities and losses could be mitigated by fish treatment with chemicals and antibiotics (Soliman et al. 2019). Although the use of antibiotics could be successful in the reduction of losses, their repeated application is often associated with potentially negative effects such as the development of antimicrobial resistance and the persistence of residues in the marine environment and seafood.

As for other vertebrate species, vaccination is a key component for sustainable and healthy farmed fish production (Miccoli et al. 2019), therefore it has been recognised as an essential prophylactic method to reduce the use of antibiotics within the aquaculture industry (Adams 2019). The incidences of antimicrobial-resistant microbes and food safety hazards could be mitigated by vaccination strategies which are highly effective and economical in protecting the health of fish from various infectious agents, ensuring environmentally friendly aquaculture and safe food supplies (AAC 2018). Vaccination should be a part of a responsible fish health management program, although it is not a short-term solution to farm sanitary problems. Based on the proper disease surveillance, fish farmers in cooperation with fish health experts should plan a specific and suitable vaccination program to obtain efficient outcomes.

Taking into consideration all the aforementioned arguments, the AdriAquaNet project aimed to the improvement of sustainability in Adriatic marine aquaculture and included fish vaccination as a key promoting measure of sustainability.

To encourage fish vaccination and to facilitate the use of vaccines in the farming of sea bass and sea bream, we addressed the project activity on two topics:

i. production of autologous vaccines against *Vibrio harveyi* and *Tenacibaculum maritimum* and laboratory and field testing of their efficacy
i. preparation and production of the document "Vaccination Strategies for Hatchery and Fish Farms"

The publication "Vaccination Strategies for Hatchery and Fish Farms" is conceived as a manual with the most important information on the role of vaccination in the management of bacterial diseases in Adriatic marine aquaculture and consists of the following chapters:

- Chapter 2 "The immune system of European sea bass (*Dicentrarchus labrax*) and gilthead seabream (*Sparus aurata*)" provides basic information on the sea bass and gilthead seabream immune system and explains which organs are involved in fish protection against pathogens, what are the protective immune mechanisms and how the fish can benefit from the vaccine administration
- Chapter 3 "Main infectious pathogens in the Adriatic Sea" is dealing with the most devastating bacterial pathogens that can affect sea bass and sea bream, giving basic information about bacteria, mechanisms of infection, ecological conditions for the disease development, clinical aspects of the disease and measures for the disease management
- Chapter 4 "Vaccination and vaccination strategy" informs readers about and gives the list of available commercial vaccines for sea bass and sea bream, the different type of vaccines and routes of vaccine administration, explains how to prepare the vaccination plan and how to perform vaccination in the hatchery, revaccination on the cages both by immersion and by injection

We hope that this document will assist the fish health managers, farmers and consultants in setting up an efficient vaccination approach, selecting the optimal vaccine and successfully preventing the spread of bacterial diseases in the Adriatic marine aquaculture. Such an achievement will contribute to the sustainability of the regional aquaculture sector, contributing to the health of the consumers and the protection of the environment.

References

Adams, A. 2019. Progress, challenges and opportunities in fish vaccine development. *Fish & shellfish immunology*, 90, 210-214.

Aquaculture Advisory Council (AAC). 2018. Relevance of the availability of effective vaccines in aquaculture. Position Paper – February 2018. pp 9

Sánchez, J. L. F., Le Breton, A., Brun, E., Vendramin, N., Spiliopoulos, G., Furones, D., & Basurco, B. 2022. Assessing the economic impact of diseases in Mediterranean grow-out farms culturing European sea bass. *Aquaculture*, 547, 737530.

Mancuso, M. 2014. Emerging Bacterial Pathologies in Mediterranean Mariculture. *Journal of Aquaculture and Research*,1, 1-2.

Miccoli, A., Saraceni, P. R., & Scapigliati, G. 2019. Vaccines and immune protection of principal Mediterranean marine fish species. *Fish & shellfish immunology*, 94, 800-809.

Pujalte, M. J., Sitjà-Bobadilla, A., Álvarez-Pellitero, P., & Garay, E. 2003. Carriage of potentially fish-pathogenic bacteria in Sparus aurata cultured in Mediterranean fish farms. *Diseases of aquatic organisms*, 54, 119-126.

Soliman, W. S., Shaapan, R. M., Mohamed, L. A., & Gayed, S. S. 2019. Recent biocontrol measures for fish bacterial diseases, in particular to probiotics, bio-encapsulated vaccines, and phage therapy. *Open veterinary journal*, 9, 190-195.

Zrnčić, S., & Pavlinec, Ž. 2020. Introduction to bacterial diseases. In. Zrnčić, S. (Ed.), Diagnostic Manual for the main pathogens in European seabass and Gilthead seabream aquaculture. *Options Mediterraneennes Serie B, Etudes et Researches* n. 75. CIHEAM, Zaragoza, pp. 63-66.; http://om.ciheam.org/om/pdf/b75/oooo7940.pdf

2. THE IMMUNE SYSTEM OF EUROPEAN SEABASS (*DICENTRARCHUS LABRAX*) AND GILTHEAD SEABREAM (*SPARUS AURATA*)

Marco Galeotti and Donatella Volpatti

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2.1. Introduction

The European seabass (*Dicentrarchus labrax*) and the gilthead seabream (*Sparus aurata*) are the most relevant marine fish species in the Mediterranean area (https://feap.info/wp-content/uploads/2022/03/production-report). Their economic importance has encouraged scientific research to focus on many aspects related to immune response and protection from diseases. For both these species, data on the genome (Tine et al. 2014, Pauletto et al. 2018) and antibodies to study immunoglobulins and leukocytes are available (Tab. 1).

SCOPUS and WOS databases revealed that immunological research has been conducted mainly by Italian academic researchers on the European seabass and by Spanish researchers on the gilthead seabream.

To date, the level of knowledge about the immune system morphology and physiology of both species is almost complete and comparable to the one gained for important freshwater teleosts like salmonids, cyprinids or zebrafish.

This chapter represents a review summary of the main morpho-functional aspects of the immune system of European seabass and gilthead seabream.

Table 1. Available antibodies anti immunoglobulins/leukocytes for European seabass (*Dicentrarchus labrax*) and gilthead seabream (*Sparus aurata*) for diagnostic or research purposes.

Target species	Gilthead seabream	European seabass
Antibodies anti immunoglobulins	• IgM (https://aquaticdiagnostics.com) • IgT (https://ximbio.com/reagent/153529/anti-igt-z55f8c3) https://bocascientific.com/	• IgM (DLIg3 mAb e pAb) • IgT pAb • IgD pAb (Ref. Prof. Scapigliati, University of Tuscia) • IgM mAb (https://aquaticdiagnostics.com)

Antibodies anti leukocytes	• G7 – specific for acidophilic granulocytes • Macrophage colony-stimulating factor receptor (Ref. Prof. Mulero, University of Murcia)	• Thymocytes (DTL15 mAb) • Lymphocytes T CD3 pAb (Ref. Prof. Scapigliati, University of Tuscia)

2.2. The organs of the immune system

Teleost fish are capable both of cellular and humoral immune response, adaptive (specific) and innate (non-specific), with some differences compared to mammals. Generally speaking, in the fish innate immune mechanisms are predominant against infectious diseases.

The main organs of the lymphohematopoietic system of fish are the thymus, the cranial portion of the kidney and the spleen (Bjørgen & Koppang 2021).

The thymus is a paired organ, located in the gill chamber, and it is responsible for the production of lymphocytes T. The function of the cranial portion of the kidney, positioned just below the vertebral column, is similar to the mammalian bone marrow. On a cellular level, it is composed of mature and immature phagocytic cells, lymphocytes and melano-macrophages. IgM synthesis takes place at this level. The spleen is a secondary lymphoid organ located in the coelomic cavity, and it contains lymphocytes, macrophages and melano-macrophages able to entrap antigens. The antigens processed at this level are considered promoters of the immunological memory.

Ontogenetic studies performed in these two marine species have described that lymphoid organs are already present at 25-30 days post-hatch (Josefsson and Tatner 1993, Quesada et al. 1994, Abelli et al. 1996, Galeotti & Beraldo, unpublished data).

Furthermore, the organs responsible for mucosal immunity are particularly important in all teleost fish. These represent a physical barrier that separates the fish from the external aqueous environment and important active immunological sites against pathogens. Mucosal associated lymphoid tissue (MALT) is the term commonly used to define this group of lymphoid associated tissues as a whole, is divided into SALT (Skin), GALT (Gut), NALT (Nasal), and GIALT (Gills) Gomez et al. 2013, Salinas 2015).

All these organs are populated by lymphocytes T and B, granulocytes, monocyte-macrophages, and eosinophilic granular cells (EGCs).

To date, mucosal immunity is currently an important subject of research studies both in European seabass and gilthead seabream species, as it is deeply involved in defensive mechanisms and its role can be modified by the microbiota (Panteli et al. 2020, Picchietti et al. 2021).

2.2.1. Leukocytes morphology

The following figure (Fig.1) illustrates the morphology of the main blood leukocyte populations in the European seabass and gilthead seabream.

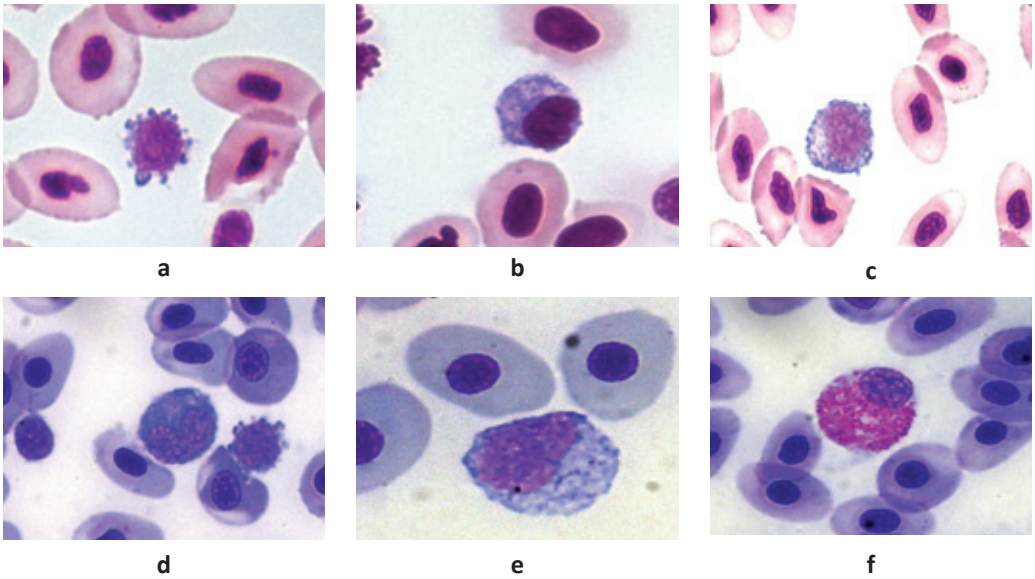

Figure 1. Blood smears stained with Giemsa. Sea bass: lymphocyte (a), monocyte (b), neutrophil granulocyte (c). Gilthead seabream: monocyte and lymphocyte (d), neutrophil granulocyte (e), acidophilic granulocyte (f).

2.3. Mechanisms of innate (non-specific) immune response

2.3.1. Complement system

The complement system includes more than 35 proteins that have a fundamental role in innate immunity, being responsible for recognizing microbial particles, and their phagocytosis or cellular lysis.

The complement system can be activated through three essential pathways: the classic pathway (CCP), the alternative pathway (ACP) and the lectin one (LCP). Teleost fish have a very efficient complement system which has been described in a limited amount of species (Boshra & Sunyer 2006), including gilthead seabream (Sunyer et al. 1997) and European sea bass (Mauri et al. 2011).

One of the most peculiar properties of this system in fish is observed in a few components like C3 and factor B. C3 protein seems to have multiple isoforms (variants) encoded by different genes (Mauri et al. 2011, Zarkardis et al. 2013): this mechanism seems to be useful to widen the spectrum of pathogens recognition.

2.3.2. Antimicrobial peptides and lysozyme

Antimicrobial peptides (AMPs) are small-sized molecules (12-50 amino acids) that play essential antimicrobial and immune-modulatory roles in the innate immune system of teleost fish.

In the last twenty years, several studies have targeted AMPs presence, their biological function and gene expression in European seabass and gilthead seabream (Cuesta et al. 2008, Terova et al. 2009, Barroso et al. 2020, 2021, Valero et al. 2020, Cervera et al. 2022). About ten different peptides, belonging to beta-defensins, NK-lysins, piscidins, hepcidins, H1-H4 histones, dicentracins, have been described in these two species.

AMPs are synthetized both in several organs (mucosae, liver, lymphatic organs) and at a cellular level (circulating monocytes and macrophages, acidophilic granulocytes, eosinophilic granular cells). Their expression and synthesis are dependent upon immune system development and exposure to viral bacterial and parasitic infections.

Together with AMPs, lysozyme has an important antibacterial role. This enzymatic molecule, mostly synthetized by hepatocytes and macrophages, has a lytic action and it is particularly effective against Gram-positive bacteria (Saurabh & Sahoo 2008, Li et al. 2021).

Several studies have focused their attention on lysozyme activity in biological matrixes like serum and skin mucus, to quantify its efficacy and modulation under different experimental conditions such as specific diets or infections, and vaccinations (Buonocore et al. 2014, Carbone et al. 2016).

2.3.3. Phagocytosis and respiratory burst

Phagocytosis in teleost fish is an essential mechanism of cell-mediated immunity capable of internalization and inactivation of pathogens, foreign particles and cellular debris (Esteban 1997). This is an innate immunity mechanism, but it can be stimulated also by the presence of antibodies.

Phagocytic cell membrane receptors can bind to ligands expressed on target pathogens; the death of pathogenic microorganisms through phagocytosis is mediated by reactive oxygen species (ROS) and nitric oxide (NO), as well as by lytic enzymes. The release of ROS molecules is defined as a respiratory burst. This rapid metabolic process can be measured experimentally and used as a parameter to quantify the effectiveness of innate cellular response (Galeotti et al. 2013).

2.3.4. Cytokines

Cytokines are an essential class of molecules involved both in the innate and adaptive immune response and in the regulation of the inflammatory process.

In teleosts, cytokine molecules have been recognized and have similar functions to the ones present in mammals (Secombes et al. 1996, Buchmann 2014). Scientific research has dedicated several studies to characterising cytokines both on gilthead seabream and European sea bass (Scapigliati et al. 2001, Castellana et al. 2013, Roman et al. 2013, Cordero et al. 2016, Reyes-Lopez et al. 2018, Miccoli et al. 2021).

2.4. Mechanisms of adaptive (specific) immune response

2.4.1. B and T lymphocytes and immunoglobulins

B and T Lymphocytes and immunoglobulins (Ig) are the main components of the adaptive (specific) immune response. From an evolutionary point of view, fish are the first vertebrates capable of specific immune response and they represent a crossing point between innate and adaptive immunity, dating back about 450 million years ago (Tort 2003, Bohem et al. 2012, Sunyer 2013, Flajnik et al. 2018).

Several studies illustrated a different level of efficacy between mammalians and fish immune systems. Lymph nodes, germinal centres and the isotypical switch from IgM to IgG seem not to be present in teleost fish.

The development of European seabass lymphocytes has been studied in detail by Dos Santos et al. (2000) and by Rombout et al. (2005). T lymphocytes in lymphatic organs develop between the 28th and 45th-day post-hatch, whereas the B lymphocytes are recognizable from the 45th to the 90th-day post-hatch. Adult levels of T and B lymphocytes are reached at about 137-145 days post-hatch.

Breuil et al. (1997), Picchietti et al. (2004) and Hanif et al. (2005) demonstrated the transfer of maternal antibodies to eggs both in gilthead seabream and European seabass, showing how broodstock vaccination can be beneficial in the transfer protection against pathogens.

Generally speaking, fish antibodies have similar functions compared to mammalians. IgM class of immunoglobulins seems to be the most relevant in seabream and seabass and they are secreted by anterior kidney plasma cells. IgM antibodies, structured as tetrameric molecules and composed of 4 monomeric units bound together by J chains, are the most common immunoglobulins present in serum, with otherwise low concentrations inside the intestinal and cutaneous mucus. IgM titre in serum increases substantially after vaccination; this process seems to be temperature-dependent.

As in zebrafish and trout, IgT antibodies class was described in gilthead seabream and European seabass (Piazzon et al. 2016, Buonocore et al. 2017); Given that their secretion is performed by plasma cells present at mucosal level (gills, skin and intestine), these antibodies are considered part of the mucosal immunity, similarly to IgA in mammalians. Finally, IgD antibodies were identified only in European seabass.

2.4.2. MHC (Major Histocompatibility Complex) molecules and other receptors

Class I and II MHC molecules are a group of glycoproteins expressed at the membrane level and able to interact with T lymphocytes triggering a defensive immune response (Wegner 2008).

Their structure, similar to the one in mammals, is composed of two sub-units α e β (Buoncore et al. 2007).

The genes capable of MHC protein expression are identified in more than 30 teleost species; they are highly polymorphic, which gives the possibility to bind a large number of different antigens. This characteristic influences many important biological traits such as the subjective resistance to diseases (Dixon et al. 2001, Buonocore et al. 2007).

As for other teleost fish, class I and II MHC were partially characterized as well and the genes responsible for the expression of MHC were recognized both in the European seabass (Buonocore et al. 2007, Pinto et al. 2013, Ratcliffe et al. 2022) and gilthead seabream (Cuesta et al. 2006, Randelli et al. 2008).

The toll-like receptors are other receptors capable of recognizing pathogen-associated molecular patterns and triggering an immune response. TLR 1, TLR 2 TLR 9 were recognized in the European seabass (Nunez Ortiz et al. 2014), whereas TLR 2, TLR 5 and TLR 22 were recognized in gilthead seabream (Munoz et al. 2014, Chen et al. 2020).

References:

Abelli, L., Gallo, V. P., Civinini, A., & Mastrolia, L. 1996. Immunohistochemical and Ultrastructural Evidence of Adrenal Chromaffin Cell Subtypes in Sea BassDicentrarchus labrax (L.). *General and comparative endocrinology*, 102, 113-122.,

Barroso, C., Carvalho, P., Carvalho, C., Santarém, N., Gonçalves, J. F., Rodrigues, P. N., & Neves, J. V. 2020. The diverse piscidin repertoire of the European sea bass (Dicentrarchus labrax): Molecular characterization and antimicrobial activities. *International journal of molecular sciences*, 21, 4613.

Bjørgen, H., & Koppang, E. O. 2022. Anatomy of teleost fish immune structures and organs. *Principles of Fish Immunology*, 1-30.

Boshra, H., Li, J., & Sunyer, J. O. 2006. Recent advances on the complement system of teleost fish. *Fish & shellfish immunology*, 20, 239-262.

Buchmann, K. 2014. Evolution of innate immunity: clues from invertebrates via fish to mammals. *Frontiers in immunology*, 5, 459.

Buonocore, F., Randelli, E., Casani, D., Costantini, S., Facchiano, A., Scapigliati, G., & Stet, R. J. 2007. Molecular cloning, differential expression and 3D structural analysis of the MHC class-II β chain from sea bass (*Dicentrarchus labrax* L.). *Fish & shellfish immunology*, 23, 853-866.

Buonocore, F., Randelli, E., Trisolino, P., Facchiano, A., de Pascale, D., & Scapigliati, G. 2014. Molecular characterization, gene structure and antibacterial activity of a g-type lysozyme from the European sea bass (*Dicentrarchus labrax* L.). *Molecular Immunology*, 62, 10-18.

Buonocore, F., Stocchi, V., Nunez-Ortiz, N., Randelli, E., Gerdol, M., Pallavicini, A., ... & Picchietti, S. 2017. Immunoglobulin T from sea bass (*Dicentrarchus labrax* L.): molecular characterization, tissue localization and expression after nodavirus infection. *BMC molecular biology*, 18, 1-14.

Carbone, D., & Faggio, C. 2016. Importance of prebiotics in aquaculture as immunostimulants. Effects on immune system of *Sparus aurata* and *Dicentrarchus labrax*. *Fish & Shellfish Immunology*, 54, 172-178.

Castellana, B., Marín-Juez, R., & Planas, J. V. 2013. Transcriptional regulation of the gilthead seabream (Sparus aurata) interleukin-6 gene promoter. *Fish & shellfish immunology*, 35, 71-78.

Cervera, L., González-Fernández, C., Arizcun, M., Cuesta, A., & Chaves-Pozo, E. 2022. Severe Natural Outbreak of Cryptocaryon irritans in Gilthead Seabream Produces Leukocyte Mobilization and Innate Immunity at the Gill Tissue. *International journal of molecular sciences*, 23, 937.

Cordero, H., Mauro, M., Cuesta, A., Cammarata, M., & Esteban, M. Á. 2016. In vitro cytokine profile revealed differences from dorsal and ventral skin susceptibility to pathogen-probiotic interaction in gilthead seabream. *Fish & shellfish immunology*, 56, 188-191.

Cuesta, A., Esteban, M. Á., & Meseguer, J. 2006. Cloning, distribution and up-regulation of the teleost fish MHC class II alpha suggests a role for granulocytes as antigen-presenting cells. *Molecular immunology*, 43, 1275-1285.

Cuesta, A., Meseguer, J., & Esteban, M. A. 2008. The antimicrobial peptide hepcidin exerts an important role in the innate immunity against bacteria in the bony fish gilthead seabream. *Molecular immunology*, 45, 2333-2342.

Dixon, B., & Stet, R. J. M. 2001. The relationship between major histocompatibility receptors and innate immunity in teleost fish. *Developmental & Comparative Immunology*, 25, 683-699.

dos Santos, N. M., Romano, N., de Sousa, M., Ellis, A. E., & Rombout, J. H. 2000. Ontogeny of B and T cells in sea bass (*Dicentrarchus labrax*, L.). *Fish & shellfish immunology*, 10, 583-596.

Esteban, M. A., & Meseguer, J. 1997. Factors influencing phagocytic response of macrophages from the sea bass (*Dicentrarchus labrax* L.): an ultrastructural and quantitative study. *The Anatomical Record: An Official Publication of the American Association of Anatomists*, 248, 533-541.

Flajnik, M. F. 2018. A cold-blooded view of adaptive immunity. *Nature Reviews Immunology*, 18, 438-453.

Galeotti, M., Romano, N., Volpatti, D., Bulfon, C., Brunetti, A., Tiscar, P. G., ... & Abelli, L. 2013. Innovative vaccination protocol against vibriosis in *Dicentrarchus labrax* (L.) juveniles: Improvement of immune parameters and protection to challenge. *Vaccine*, 31, 1224-1230.

Gomez, D., Sunyer, J. O., & Salinas, I. 2013. The mucosal immune system of fish: the evolution of tolerating commensals while fighting pathogens. *Fish & shellfish immunology*, 35, 1729-1739.

Hanif, A., Bakopoulos, V., Leonardos, I., & Dimitriadis, G. J. 2005. The effect of sea bream (*Sparus aurata*) broodstock and larval vaccination on the susceptibility by *Photobacterium damsela* subsp. *piscicida* and on the humoral immune parameters. *Fish & shellfish immunology*, 19, 345-361.

https://feap.info/wp-content/uploads/2022/03/production-report

https://aquaticdiagnostics.com/

https://ximbio.com/reagent/153529/anti-igt-z55f8c3

https://bocascientific.com/

https://aquaticdiagnostics.com/

Jósefsson, S., & Tatner, M. F. 1993. Histogenesis of the lymphoid organs in sea bream (*Sparus aurata* L.). *Fish & Shellfish Immunology*, 3, 35-49.

Li, J., Jiang, X., Li, H., Gelinsky, M., & Gu, Z. 2021. Tailoring materials for modulation of macrophage fate. *Advanced Materials*, 33, 2004172.

Mauri, I., Roher, N., MacKenzie, S., Romero, A., Manchado, M., Balasch, J. C., ... & Tort, L. 2011. Molecular cloning and characterization of European seabass (*Dicentrarchus labrax*) and Gilthead seabream (*Sparus aurata*) complement component C3. *Fish & Shellfish Immunology*, 30, 1310-1322.

Miccoli, A., Buonocore, F., Picchietti, S., & Scapigliati, G. 2021. The sea bass *Dicentrarchus labrax* as a marine model species in immunology: Insights from basic and applied research. *Aquaculture and Fisheries*. https://doi.org/10.1016/j.aaf.2021.09.003

Ortiz, N. N., Gerdol, M., Stocchi, V., Marozzi, C., Randelli, E., Bernini, C., ... & Scapigliati, G. 2014. T cell transcripts and T cell activities in the gills of the teleost fish sea bass (*Dicentrarchus labrax)*. *Developmental & Comparative Immunology*, 47, 309-318.

Panteli, N., Mastoraki, M., Lazarina, M., Chatzifotis, S., Mente, E., Kormas, K. A., & Antonopoulou, E. 2021. Configuration of gut microbiota structure and potential functionality in two teleosts under the influence of dietary insect meals. *Microorganisms*, 9, 699.

Pauletto, M., Manousaki, T., Ferraresso, S., Babbucci, M., Tsakogiannis, A., Louro, B., ... & Bargelloni, L. 2018. Genomic analysis of *Sparus aurata* reveals the evolutionary dynamics of sex-biased genes in a sequential hermaphrodite fish. *Communications biology*, 1, 1-13.

Piazzon, M. C., Galindo-Villegas, J., Pereiro, P., Estensoro, I., Calduch-Giner, J. A., Gómez-Casado, E., ... & Pérez-Sánchez, J. 2016. Differential modulation of IgT and IgM upon parasitic, bacterial, viral, and dietary challenges in a perciform fish. *Frontiers in immunology*, 7, 637.

Picchietti, S., Buonocore, F., Guerra, L., Belardinelli, M. C., De Wolf, T., Couto, A., ... & Scapigliati, G. 2021. Molecular and cellular characterization of European sea bass CD3ε+ T lymphocytes and their modulation by microalgal feed supplementation. *Cell and Tissue Research*, 384, 149-165.

Picchietti, S., Taddei, A. R., Scapigliati, G., Buonocore, F., Fausto, A. M., Romano, N., ... & Abelli, L. 2004. Immunoglobulin protein and gene transcripts in ovarian follicles throughout oogenesis in the teleost *Dicentrarchus labrax*. *Cell and tissue research*, 315, 259-270.

Pinto, R. D., Randelli, E., Buonocore, F., Pereira, P. J., & dos Santos, N. M. 2013. Molecular cloning and characterization of sea bass (*Dicentrarchus labrax*, L.) MHC class I heavy chain and β2-microglobulin. *Developmental & Comparative Immunology*, 39, 234-254.

Quesada, J., Villena, M. I., & Navarro, V. 1994. Ontogeny of the sea bass spleen (*Dicentrarchus labrax)*: a light and electron microscopic study. *Journal of morphology*, 221, 161-176.

Randelli, E., Scala, V., Casani, D., Costantini, S., Facchiano, A., Mazzini, M., ... & Buonocore, F. 2008. T cell receptor beta chain from sea bream (*Sparus aurata*): molecular cloning, expression and modelling of the complexes with MHC class I. *Molecular Immunology*, 45, 2017-2027.

Ratcliffe, F. C., Garcia de Leaniz, C., & Consuegra, S. 2022. MHC class I-α population differentiation in a commercial fish, the European sea bass (*Dicentrarchus labrax*). *Animal Genetics*, 53, 340-351

Reyes-López, F. E., Aerts, J., Vallejos-Vidal, E., Ampe, B., Dierckens, K., Tort, L., & Bossier, P. 2018. Modulation of innate immune-related genes and glucocorticoid synthesis in gnotobiotic full-sibling European sea bass (*Dicentrarchus labrax*) larvae challenged with *Vibrio anguillarum*. *Frontiers in immunology*, 9, 914.

Román, L., Real, F., Padilla, D., El Aamri, F., Déniz, S., Grasso, V., & Acosta, F. 2013. Cytokine expression in head-kidney leucocytes of European sea bass (*Dicentrarchus labrax* L.) after incubation with the probiotic *Vagococcus fluvialis* L-21. *Fish & shellfish immunology*, 35, 1329-1332.

Rombout, J. H. W. M., Huttenhuis, H. B. T., Picchietti, S., & Scapigliati, G. 2005. Phylogeny and ontogeny of fish leucocytes. *Fish & shellfish immunology*, 19, 441-455.

Salinas, I. 2015. The mucosal immune system of teleost fish. *Biology*, 4, 525-539.

Saurabh, S., & Sahoo, P. K. 2008. Lysozyme: an important defence molecule of fish innate immune system. *Aquaculture research*, 39, 223-239.

Scapigliati, G., Buonocore, F., Bird, S., Zou, J., Pelegrin, P., Falasca, C., ... & Secombes, C. J. 2001. Phylogeny of cytokines: molecular cloning and expression analysis of sea bass *Dicentrarchus labrax* interleukin-1β. *Fish & Shellfish Immunology*, 11, 711-726.

Secombes, C. J. 1996. The nonspecific immune system: cellular defenses. *The fish immune system: organism, pathogen and environment*, 15, 63-103.

Sunyer, J. O. 2013. Fishing for mammalian paradigms in the teleost immune system. *Nature immunology*, 14, 320-326.

Sunyer, J. O., Tort, L., & Lambris, J. D. 1997. Structural C3 diversity in fish: characterization of five forms of C3 in the diploid fish *Sparus aurata*. *The Journal of Immunology*, 158, 2813-2821.

Terova, G., Forchino, A., Rimoldi, S., Brambilla, F., Antonini, M., & Saroglia, M. 2009. Bio-Mos®: an effective inducer of dicentracin gene expression in European sea bass (*Dicentrarchus labrax*). *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 153, 372-377.

Tine, M., Kuhl, H., Gagnaire, P. A., Louro, B., Desmarais, E., Martins, R. S., ... & Reinhardt, R. 2014. European sea bass genome and its variation provide insights into adaptation to euryhalinity and speciation. *Nature communications*, 5, 1-10.

Tort, L., Balasch, J. C., & Mackenzie, S. 2003. Fish immune system. A crossroads between innate and adaptive responses. *Inmunología*, 22, 277-286.

Valero, Y., Arizcun, M., Cortes, J., Ramirez-Cepeda, F., Guzman, F., Mercado, L., ... & Cuesta, A. 2020. NK-lysin, dicentracin and hepcidin antimicrobial peptides in European sea bass. Ontogenetic development and modulation in juveniles by nodavirus. *Developmental & Comparative Immunology*, 103, 103516.

Wegner, K. M. 2008. Historical and contemporary selection of teleost MHC genes: did we leave the past behind?. *Journal of Fish Biology*, 73, 2110-2132.

3. THE MAIN BACTERIAL PATHOGENS IN THE ADRIATIC SEA

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Recently published reports on the disease surveillance in the Mediterranean basin show that bacterial infections are predominantly noticed (75.0%) in European sea bass farming, whereas parasitic infections (57.0%) are the most frequently reported infections in gilthead sea bream farming (Muniesa et al. 2020). The same reports showed that vibriosis caused by *Vibrio* sp. is the most frequently reported bacterial disease in sea bass and sea beam during the on-growing phase, followed by tenacibaculosis caused by *Tenacibaculum maritimum* and photobacteriosis caused by *Photobacterium damselae* subsp. *piscicida*. Vibriosis is reported from all fish stages throughout the production chain, whereas tenacibaculosis and photobacteriosis seem to be more problematic for the on-growing phases. Moreover, seabass is more susceptible compared to gilthead sea bream to the above-mentioned pathogens (Rigos et al. 2021). Currently, no specific reports on the prevalence of bacterial diseases in the Adriatic Sea are available but the data obtained from farms situated in this area reveal that the real situation is very similar if not identical to that reported in the cited documents.

To ensure that farmed fish are kept in good health conditions, all people involved in sea bass and sea bream health management should have basic information about the diseases, therefore we are providing here a short review about each of the aforementioned bacterial diseases.

References

Muniesa, A., Basurco, B., Aguilera, C., Furones, D., Reverté, C., Sanjuan-Vilaplana, A., M. Dverdal Jansen, E. Brun & Tavornpanich, S. 2020. Mapping the knowledge of the main diseases affecting sea bass and sea bream in Mediterranean. *Transboundary and Emerging Diseases*, 67, 1089-1100.

Rigos, G., Kogiannou, D., Padrós, F., Cristofol, C., Florio, D., Fioravanti, M., & Zarza, C. 2021. Best therapeutic practices for the use of antibacterial agents in finfish aquaculture: A particular view on European seabass (*Dicentrarchus labrax*) and gilthead seabream (*Sparus aurata*) in Mediterranean aquaculture. *Reviews in Aquaculture*, 13, 1285-1323.

3.1. Vibriosis caused by *Vibrio anguillarum*

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3.1.1. Pathogen

Vibriosis caused by *Vibrio anguillarum* affects a variety of different marine and brackish water fish and mollusc species and it was described for the first time in 1718 in Italy as a "red pest" after the survey of significant mortalities in eels (Austin and Austin 2007). Currently, the disease is spread worldwide, as many reports have been documented in more than 50 marine and freshwater fish species (Toranzo et al. 2004).

The pathogen responsible for the disease called "*pestis rubra anguillarum*" or "*erysipelosis anguillarum*" was initially denominated as *Bacillus anguillarum.* Later on, the same disease was observed in eels from the Baltic Sea and the isolated pathogen was named *Vibrio anguillarum.* Thanks to the development of specific molecular tools and based on sequencing data, MacDonell and Colwell (1985) reclassified the pathogen as *Listonella anguillarum* (Wiik et al. 1995) and it was officially excluded from the Vibrionaceae family taxon. Only three bacterial species were classified within the genus *Listonella* – *L. anguillarum*, *L. damselae* and *L. pelagius* – until they were later reclassified under the *Vibrio* genus (Dikow 2011, Thompson et al. 2011). Today, the accepted classification of *V. anguillarum* appears to be *Vibrio* (*Listonella*) *anguillarum* as controversy among preference and related studies remain prevalent (Hickey & Lee 2018).

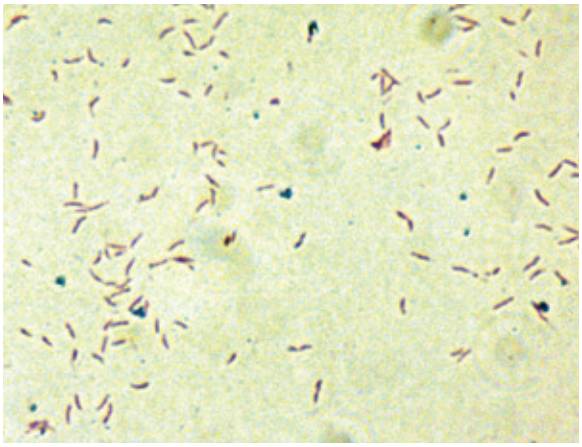

Figure 2. Comma shaped *Vibrio anguillarum* in a Gram-stained smear.

Figure 3. Typical yellow colonies of *V. anguillarum* on TCBS agar.

V. anguillarum is a Gram-negative, comma-shaped, rod 0.3 to 0.5 µm in width, and 1.0 to 3.5µm long (Fig. 2), non-spore-forming, halophilic, a facultative anaerobic bacterium with single monotrichous sheathed polar flagella (Actis et al. 1999). It grows on media containing 1.5 to 2% NaCl at 15 to 30 °C and it produces creamy, yellow round-shaped colonies on the *Vibrio*-selective thiosulfate citrate bile sucrose (TCBS) agar (Fig. 3), indicating fermentation of sucrose. Until now, 23 different *V. anguillarum* serotypes (O1-O23) have been identified but only the serotypes O1, O2 and, to a lesser extent, O3 showed pathogenicity to fish species whereas the other serotypes commonly isolated from environmental samples resulted in no pathogenic (Pedersen et al. 1999).

3.1.2. Infection and ecological factors

The infection route of *V. anguillarum* is still the subject of debate; bacteria are ingested by fish through contaminated food or water, they survive the gastric low pH, then they enter into the gut where they adhere to the intestinal epithelium and proliferate, finally, they enter into the blood causing septicaemia (Grisez et al. 1996). Another route of entry is the penetration of bacteria into an injured skin or damaged mucus layer. The bacteria spread is horizontal from infected fish to healthy fish, but also through contaminated feed, water or equipment. *V. anguillarum* is a natural inhabitant of the marine environment, therefore it could survive in the sediment for up to 50 months

The disease occurs commonly when the temperatures are high. Predisposing factors for the occurrence of disease outbreaks are also poor water oxygen saturation, low water exchange and stress due to the increase or decrease of water temperature, high population density, handling, etc. (Le Breton 1996, Frans et al. 2011). The presence of heavy metals, particularly copper and iron, contributes to the exacerbation of the disease. In particular, high concentration and prolonged exposure to copper increase the fish's susceptibility to vibriosis (Austin & Austin 1993). In the Adriatic Sea, the vibriosis development depends on the season since it is widely documented that the acute and subacute forms predominantly occur during the spring and autumn whereas the chronic forms mostly occur during the winter.

3.1.3. Clinical aspects of the disease

The acute form of vibriosis generally affects the young fish, usually without any symptoms and mortalities up to 80% (Frerichs and Roberts 1989). **The acute and subacute form** of the disease is characterized by lethargy, anorexia and darkening of the skin usually as the first symptoms (Fig. 4), followed by erythema around the mouth and the vent, on the bases of fins, oedematous lesions in the skin, ulceration and bleeding lesions and bleeding on the head, operculum, vent, with pale gills and haemorrhages

(Fig. 5). On autopsy, the bleedings on the liver and posterior part of the intestine and rarely in the stomach are present (Fig. 6). **The chronic form** is characterized by large granulating lesions penetrating deep into the muscle and progressing to ulceration (Fig. 7), severe anaemia of the gills and grey corneal opacity (Haenen et al. 2014).

Figure 4. An increase in skin pigmentation and swimming separated from the shoal are the first symptoms of vibriosis caused by *V. anguillarum*.

Figure 5. Extensive haemorrhages on the head, skin and fins in sea bass affected by a subacute form of vibriosis caused by *V. anguillarum*.

Figure 6. Haemorrhages on the liver, stomach and intestine are often visible in sea bass affected by an acute form of vibriosis caused by *V. anguillarum*.

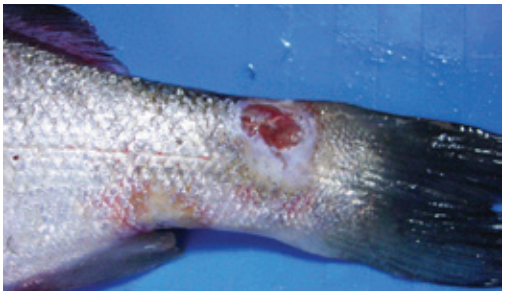

Figure 7. A granulating lesion was observed in sea bass affected by a chronic form of vibriosis caused by *V. anguillarum*.

3.1.4. Disease management

Several commercial vaccines have been developed to protect fish against vibriosis, consisting mostly of inactivated both *V. anguillarum* serotypes O1 and O2 (Haenen et al. 2014). Their efficiency depends on the administration route: i) the intraperitoneal administration is the most effective but time-consuming, needs the engagement of many people, and induces the formation of granulomas, inflammation and pigmentation ii) the immersion procedure is often preferred despite a shorter duration of immunity iii) oral vaccination is the least effective due to the vaccine degradation during its passage through the intestinal tract.

An efficient method of vibriosis prevention should include GAP, implementation of hygienic and biosecurity measures, avoiding stress during fish manipulation, and immunostimulation. Some alternative prophylactic measures such as the dietary administration of probiotic bacterial isolates, prebiotics, algae, yeast extracts or other immunostimulants such as marine natural products (MNPs) are giving promising results, being able to improve the fish immune responses (Rodrigues-Estrada et al. 2008).

In some cases, the application of antimicrobials is inevitable and the treatment with flumequine, potentiated sulphonamides and oxytetracycline in the feed is effective in reducing the fish losses in mariculture. However, it is recommended to make an early diagnosis and an antibiogram to test the isolated bacterial strain for antibiotic susceptibility (Haenen et al. 2014).

References

Actis, L. A., Tolmasky, M. E., & Crosa, J. H. 2011. *Vibriosis* in *Fish diseases and disorders. Volume 3: viral, bacterial and fungal infections*, (Ed. 2). CABI International, Wallingford, UK. pp. 570-605.

Austin, B., & Austin, D. A. 1993. *Bacterial fish pathogens: diseases in farmed and wild fish*, (Ed. 2). Ellis Horwood, London. pp. 265-307.

Austin, B., & Austin, D. A. 2007. *Bacterial fish pathogens: diseases in farmed and wild fish*, (Ed. 4). Springer-Praxis Publishing, Ltd., United Kingdom

Dikow, R.B. 2011. Systematic relationships within Vibrionaceae (Bacteria: Gammaproteobacteria): steps toward a phylogenetic taxonomy. *Cladistics*, 27, 9– 28.

Frans, I., Michiels, C. W., Bossier, P., Willems, K. A., Lievens, B., & Rediers, H. 2011. Vibrio anguillarum as a fish pathogen: virulence factors, diagnosis and prevention. *Journal of fish diseases*, 34, 643-661.

Frerichs, G. N., & Roberts, R. J. 1989. The bacteriology of teleosts. *Fish pathology*, 289-319.

Grisez, L., Sorgeloos, P., & Ollevier, F. 1996. Mode of infection and spread of Vibrio anguillarum in turbot *Scophthalmus maximus* larvae after oral challenge through live feed. *Diseases of aquatic organisms*, 26, 181-187.

Haenen, O. L. M., Fouz Rodríguez, B., Amaro González, C., Isern, M. M., Mikkelsen, H., Zrnčić, S.,& Dalsgaard, I. 2014. Vibriosis in aquaculture. 16th EAFP Conference, Tampere, Finland, 4th September 2013. *Bulletin of the European Association of Fish Pathologists,* 34*,* 138-147.

Hickey, M. E., & Lee, J. L. 2018. A comprehensive review of *Vibrio* (*Listonella*) *anguillarum*: ecology, pathology and prevention. *Reviews in Aquaculture*, 10, 585-610.

Le Breton, A. 1996. An overview of the main infectious problems in cultured seabass *Dicentrarchus labrax* and seabream *Sparus aurata*: solutions. In *International workshop on ''sea bass and sea bream culture: problems and prospects''. European Aquaculture Society* (pp. 67-86).

MacDonell, M. T., & Colwell, R. R. 1985) Phylogeny of the Vibrionaceae, and recommendation for two new genera, *Listonella* and *Shewanella*. *Systematic and applied microbiology*, 6, 171-182.

Pedersen, K., Grisez, L., Houdt, R. V., Tiainen, T., Ollevier, F., & Larsen, J. L. 1999. Extended serotyping scheme for *Vibrio anguillarum* with the definition and characterization of seven provisional O-serogroups. *Current microbiology*, 38, 183-189.

Rodrigues-Estrada, U., Satoh, S., Haga, Y., Fushimi, H., & Sweetman, J. 2008. Studies of the effects of mannan-oligosaccharides, *Enterococcus faecalis*, and poly hydrobutyric acid as immune stimulant and growth promoting ingredients in rainbow trout diets. In *5th World Fisheries Congress, Yokohama, Japan, October* (pp. 20-25).

Thompson, F.L., C.C. Thompson, G.M. Dias, H. Naka, C. Dubay, J.H. Crosa. 2011. The genus *Listonella* MacDonell and Colwell 1986 is a later heterotypic synonym of the genus *Vibrio* Pacini 1854 (approved lists 1980) – a taxonomic opinion. *International Journal of Systematic and Evolutionary Microbiology*, 61, 3023-3027.

Toranzo, A. E. 2004. Report about fish bacterial diseases. *Mediterranean Aquaculture Laboratories, ed. Alvarez-Pellitero P, Barja JL, Basurco B, Berthe F, Toranzo AE*, 49-89.

3.2. Vibriosis caused by *Vibrio harveyi*

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3.2.1. Pathogen

Vibrio harveyi clade represents a group of very important pathogens of aquatic animals with eleven closely related bacterial species: *V. harveyi, V. alginolyticus, V. parahaemolyticus, V. campbellii, V. rotiferianus, V. mytili, V. natriegens, V. azureus, V. sagamiensis, V. owensii*, and *V. jasicida* that have a high phenotypic and genotypic homology (Urbanczyk et al. 2013). All of them are commonly found in marine and estuarine water and sediments (Hernandez et al. 2004).

V. harveyi was initially described as a cause of mass mortalities in shrimp hatcheries, then it was isolated from many disease outbreaks in different fish species reared in the subtropical region such as groupers, barramundi, flatfish, pompano and it is considered as the most important pathogen in Chinese marine aquaculture (Karunasagar et al. 1994; Lavilla-Pitogo et al. 1998; Qin et al., 2006; Tendencia 2002; Pakingking et al. 2018). In the last decade, *V. harveyi* more often causes serious losses in Mediterranean and Adriatic aquaculture during the summer months (Zupicic et. 2019).

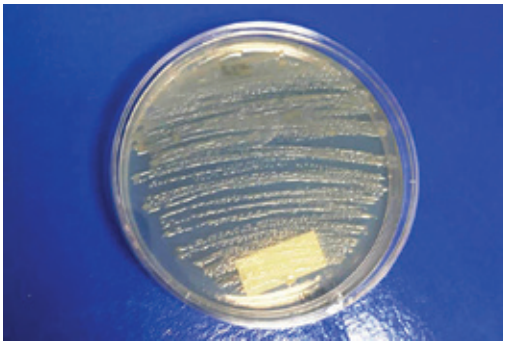

Figure 8. Typical colonies of *Vibrio harveyi*

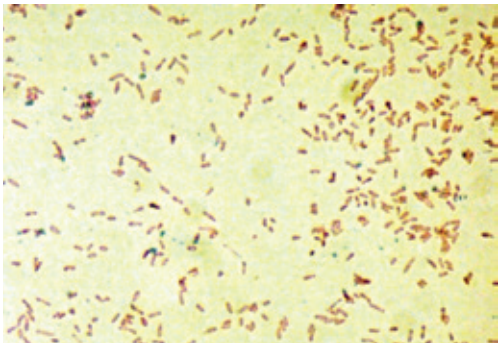

Figure 9. *V. harveyi* in a Gram-stained smear

V. harveyi is a Gram-negative (Fig. 8, 9), halophilic, aerobic or facultatively anaerobic bacterium that has similar growth requirements to *V. anguillarum.* It is an opportunistic bacterium and non-pathogenic strains are often found in the normal flora of the host and environment, although highly pathogenic strains have been sometimes isolated (Pretto 2018). In the Mediterranean basin, different serotypes have been determined but three are dominant, and among them, a new “emerging” very pathogenic serovar has been detected in Spain and the Adriatic sea. However, some of the *V. harveyi* isolates are not belonging to any of these three defined serovars (Amaro et al. 2020).

3.2.2. Infection and ecological factors

The main factors of *V. harveyi* virulence are the bacterial flagellum which enables mobility, lytic enzymes, capsule formation, siderophores which assist iron-binding, hydrophobic surface antigens, and the ability to adhere and infect the epithelial host cells (Wang and Leung 2000; Ruwandeepika et al. 2012). The production of biofilm is the mechanism of *antibiotic resistance,* and the ability of bacteria to extract iron from host cells is crucial for their survival. Moreover, the intercellular communication among bacteria within host cells allows them to act as a group and it is of crucial importance for their virulence (Themptander 2005).

Pathogenesis is based on chemotaxis, which enables the pathogen to enter the host tissue, activate the iron sequestering mechanism and produce extracellular toxins causing clinical symptoms in fish. The infection starts when bacteria enter the fish intestine or through the bloodstream, then they colonize the organs causing septicaemia and death of infected fish (Thompson et al. 2004). The disease is transferred horizontally from diseased fish to healthy fish.

It has been proved that salinity and sea water temperature are crucial for the pathogenicity of *V. harveyi*. In the Adriatic region, the disease usually occurs during the summer months when the sea temperatures are high.

3.2.3. Clinical aspect of the disease

Figure 10. Erosions on the skin of the head and haemorrhages on the trunks and fin basis in sea bass affected caused by *V. harveyi*

Figure 11. Congestion on the liver and serocatharal enteritis in sea bass affected caused by *V. harveyi*

Figure 12. Symptoms provoked by i/p infection with *V. harveyi* in sea bass

Prvi simptomi bolesti su letargija i gubitak apetita, nakon čega slijedi depigmentacija, erozije kože, krvarenja na osnovi peraja, nekroze i ulceracije, blijede škrge, krvarenja po škržnom epitelu. U uznapredovalom stadiju bolesti mogu se uočiti živčani simptomi karakterizirani nekoordiniranim plivanjem zajedno s keratitisom, zamućenošću rožnice te egzoftalmijom. S obzirom da ti klinički simptomi sliče onima koji se javljaju tijekom infekcije virusom nervne nekroze (NNV) ili betanodaviroze, ovu je virusnu bolest potrebno diferencijalno isključiti iz dijagnostike. Živčani simptomi se javljaju zbog encefalne kongestije.

Kod obdukcije su u tjelesnoj šupljini prisutna krvarenja te eksudat ili ascites. Vidljiva je fokalna kongestija jetre i petehijalna krvarenja po jetri, a jedan od tipičnih simptoma je serozni do serokataralni enteritis, nekroza crijeva i lumen crijeva ispunjen bijelim do žućkastim eksudatom (Zhang i Austin 2000).

3.2.4. Disease management

No effective commercial vaccines are currently available, although several scientific papers describe promising results after experimental vaccination with different types of vaccines.

For this reason, the only effective management strategies to prevent the spread of vibriosis caused by *V. harveyi* in farms are the application of good aquaculture practice (GAP) and fish treatment with antimicrobials. Regarding this aspect, most of the Croatian isolates of *V. harveyi* resulted susceptible to oxytetracycline, flumequine, florfenicol and co-trimoxazole.

Recently, a certain advantage has been achieved in some experiments with phage therapy, that demonstrated the efficacy of lytic bacteriophage on brine shrimp *Artemia* infected with *V. harveyi* was demonstrated (Misol et al. 2020).

References

Amaro, C., Fouz, B., Sanjuan, E., Romalde., J.L. 2020. Vibriosis in *Climate Change and Infectious Fish Diseases.* (Woo, P. T. K., J. A. Leong, K. Buchmann, Eds.). CAB International, Wallingford, UK. pp. 182-210.

Hernández-Castro, R., Martínez-Chavarría, L., Díaz-Avelar, A., Romero-Osorio, A., Godínez-Reyes, C., Zavala-González, A., & Verdugo-Rodríguez, A. 2005. Aerobic bacterial flora of the nasal cavity in Gulf of California sea lion (Zalophus californianus) pups. *The Veterinary Journal*, 170, 359-363.

Karunasagar, I., Pai, R., Malathi, G. R., & Karunasagar, I. 1994. Mass mortality of *Penaeus monodon* larvae due to antibiotic-resistant *Vibrio harveyi* infection. *Aquaculture*, 128, 203-209.

Lavilla-Pitogo, C. R., Leaño, E. M., & Paner, M. G. 1998. Mortalities of pond-cultured juvenile shrimp, *Penaeus monodon*, associated with dominance of luminescent vibrios in the rearing environment. *Aquaculture*, 164, 337-349.

Misol, G. N., Kokkari, C., & Katharios, P. 2020. Biological and genomic characterization of a novel jumbo bacteriophage, vB_VhaM_pir03 with broad host lytic activity against *Vibrio harveyi*. *Pathogens*, 9, 1051.

Pakingking Jr, R., Bautista, N. B., Catedral, D., & de Jesus-Ayson, E. G. 2018. Characterisation of *Vibrio* isolates recovered from the eyes of cage-cultured pompano (*Trachinotus blochii*) infested with caligid parasites (*Lepeophtheirus spinifer*). *European Association of Fish Pathologists Bulletin*, 38, 35-41.

Pretto, T. 2018. *Vibriosis caused by Vibrio harveyi: studies on pathogenesis and vaccine efficacy in European sea bass (Dicentrarchus labrax)* (Doctoral dissertation, Doctoral dissertation thesis). Alma Mater Studiorum, University of Bologna, Italy.

Yingxue, Q., Jun, W., Yongquan, S., Dexiang, W., & Xinzhong, C. 2006. Studies on the pathogenic bacterium of ulcer disease in *Epinephelus awoara*. *Acta Oceanologica Sinica*, 1, 154-159.

Tendencia, E. A. 2002. *Vibrio harveyi* isolated from cage-cultured seabass Lates *calcarifer* Bloch in the Philippines. *Aquaculture Research*, 33, 455-458.

Themptander, K. 2005. *Detection and characterisation of Vibrio harveyi isolates* (Doctoral dissertation, Doctoral dissertation thesis). Medical Faculty University of Upsala.

Thompson, F. L., Iida, T., & Swings, J. 2004. Biodiversity of vibrios. *Microbiology and molecular biology reviews*, 68, 403-431.

Urbanczyk, H., Ogura, Y., & Hayashi, T. 2013. Taxonomic revision of Harveyi clade bacteria (family Vibrionaceae) based on analysis of whole genome sequences. *International journal of systematic and evolutionary microbiology*, 63, 2742-2751.

Wang, X. H., & Leung, K. Y. 2000. Biochemical characterization of different types of adherence of Vibrio species to fish epithelial cells. *Microbiology*, 146, 989-998.

Darshanee Ruwandeepika, H. A., Sanjeewa Prasad Jayaweera, T., Paban Bhowmick, P., Karunasagar, I., Bossier, P., & Defoirdt, T. 2012. Pathogenesis, virulence factors and virulence regulation of vibrios belonging to the Harveyi clade. *Reviews in Aquaculture*, 4, 59-74.Zhang, X. H. and B. Austin. 2000. Pathogenicity of *Vibrio harveyi* to salmonids. *Journal of Fish Diseses*, 23, 93-102.

Zupičić, I. G., Pavlinec, Ž., Oraić, D., & Zrnčić, S. 2019. Emerging vibriosis of Mediterranean fish caused by the bacterial species Vibrio harveyi: an overview. *Veterinarska stanica*, 50, 455-463.

3.3. Photobacteriosis caused by *Photobacterium damselae subsp. piscicida*

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University of Udine Dept. of Agricultural, Food, Environmental and Animal Sciences

3.3.1. Pathogen

Photobacteriosis caused by *Photobacterium damselae* subsp. *piscicida*, formerly called pasteurellosis or pseudotuberculosis, is a septicemic disease that was first reported in 1953 in the USA and then spread rapidly in Japan and also in Europe where it was found in the 1990s in several countries of the Mediterranean basin. Currently, it is still considered one of the most important bacterial diseases of mariculture, having a worldwide distribution and affecting a large number of sea water and brackish fish species, both farmed and wild (it survives in fresh water for about 48 hours and in brackish water for 3-5 days) with significant mortality rates and consequent economic losses in mariculture facilities. The most affected fish species are seriola (*Seriola quinqueradiata*) in Japan, common seabream (*Pagrus pagrus*), red seabream (*Pagrus major*), sea bass (*Dicentrarchus labrax*), scallop (*Argyrosomus regius*), sole (*Solea* spp.), striped bass (*Morone saxatilis*), white perch (*Morone americana*) and a hybrid of striped bass and white bass (*Morone saxatilis* (*Morone chrysops*)) in the USA, sea bream and sea bass in many European countries including France, Italy, Croatia, Malta, Spain, Portugal, Greece, Israel, and Turkey (Andreoni & Magnani 2014).

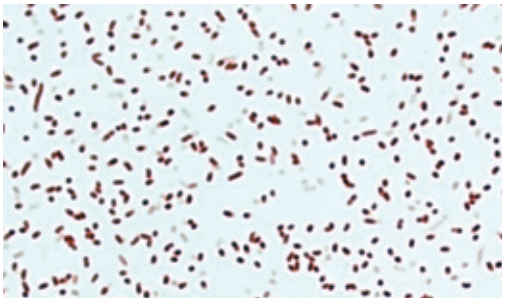

Figure 13. *Photobacterium damselae* subsp. *piscicida* colony smear coloured with Giemsa.

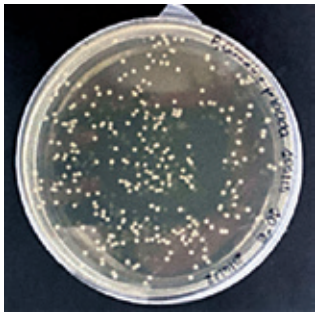

Figure 14. Typical white and translucent colonies of *Photobacterium damselae* subsp. *piscicida* isolated in sea bass. The colonies grow on trypticase soy agar (TSA) or blood agar.

P. damselae subsp. *piscicida* (Phdp) is a Gram-negative, halophilic, asporogenic, facultatively anaerobic bacterium, member of the Vibrionaceae family (Fig. 13, 14). It was first isolated in perch (*Morone americanus*) and perch (*Morone saxatilis*) by Sniezsko (1964), it was subsequently classified by Jansen and Surgalla (1968), then it was definitively identified by Gauthier et al. (1995).

3.3.2. Infection, pathogenesis and environmental factors

The infections caused by *P. damselae* subsp. *piscicida* developed by the horizontal transmission of bacteria from infected fish to healthy fish or through contaminated water, food or equipment. The penetration of bacteria into the host occurs through the skin, gills and intestine. Bacteria adhere to skin, gills or intestinal epithelial cells thanks to their robust lipopolysaccharide capsule, which facilitates their adhesion as observed in the intestine of the sea bream (Galeotti et al 1995, 1996, Magariños et al. 1996a, 1996b, Romalde 2002).

The incubation period of the disease can last from 48 hours to 4 days, then strong septicemia develops and bacteria spread to all blood districts and reach various organs including the liver, spleen, kidney and heart. In the spleen, bacteria are rapidly phagocytosed by macrophages and neutrophils but remain alive inside them for at least 7 days thanks to the lipopolysaccharide capsule, which does not allow the cells to carry out the killing and causes their lysis. Macrophages often carry bacteria in the blood and other organs, protecting them from the host specific and non-specific immune defences as well as from exogenous antimicrobial agents including antibiotics through a Trojan horse-like mechanism (Galeotti et al. 1995, 1996; Romalde, 2002, Barnes and Ellis 2004, Jung et al. 2008, Acosta et al. 2009). In addition, resistant bacterial emboli to the microbicidal action of the immune components contained in the serum have been observed in diseased fish, which are carried by the blood together with the macrophages loaded with bacteria and reach the branchial circulation, where they can block at the base of the capillaries of the primary lamellae and cause serious ischemic lesions and necrotic effects. This pathogenetic mechanism is considered the main cause of death by asphyxia in fish infected with Phdp (Galeotti et al. 1995, 1996).

Other pathogenic actions of Phdp are the ability to sequester iron through high-affinity siderophores or by acquiring it from hemin and haemoglobin (Magariños et al. 1994, Jung et al. 2007) and the secretion of extracellular products (ECPs) with hemolytic, cytotoxic and phospholipase activity, which are responsible for the damage to infected cells, the consequent release of bacteria and the colonization of adjacent cells (Bakopoulos et al. 2002, 2004). In particular, the secretion of a plasmid-encoded exotoxin (AIP56) that activates the apoptosis of macrophages and neutrophils seems important. (do Vale et al. 2005). Recently, Phdp has been shown to induce an up-regulation of genes with suppressive functions resulting in a suppression of the host immunity (Pellizzari et al. 2013).

In general, the disease develops when the water temperature is high (above 23 ° C), the salinity is between 20‰ and 30‰, the oxygen concentration is low and the water quality is poor. Predisposing factors that can allow the spread of disease are the excessive density of fish in the farmed tanks/cages, the appearance of environmental stress (sudden increase or decrease in water temperature, excessive manipulation), or altered water physicochemical parameters (altered temperature, salinity and oxygen saturation, eutrophication, presence of pollutants) (Micoli et al. 2019).

Different susceptibility of fish to disease has also been demonstrated, depending on fish age. Larvae and juveniles are more susceptible to infections caused by Phdp than adults over 50 g in size and show mortality up to 90-100% during acute infections. This evidence is due to the higher functionality of macrophages and neutrophils in adult fish, which can more efficiently phagocytize and kill the bacteria (Romalde 2002, Andreoni and Magnani 2014).

3.3.3. Clinical and anatomopathological aspects of disease

The disease commonly occurs in a **hyperacute and acute form** in the larval and juvenile stages. Fish are hyper-pigmented, lethargic, with swimming imbalances and swimming on the surface, decreased nutrition and increased respiratory rate. With the disease progression, anorexia, lethargy, darkening of the skin and sometimes skin erosions are observed. The gills become pale, with hypersecretion and necrotic areas. Areas with slight vessel congestion and erosive events appear on the skin, especially affecting the attachment of the fins, on the muzzle or near the anus. Dead fish are found at the bottom of the tanks, with little injury. Upon opening the abdominal cavity, slight diffuse haemorrhages are evident and the spleen appears splenomegalic, scattered with small whitish areas, tendentially circular, undetected, which constitute necrotic foci (Fig. 15, 16). The kidney appears clear (Fig. 15). Histological observations reveal the spleen is covered with numerous bacterial colonies surrounded by macrophages filled with bacteria (Fig. 17, 18), which appear strongly positive after immunohistochemistry (IHC) with a specific antibody (Fig. 19, 20). Around the bacterial colonies and macrophages, the presence of abundant acidophilic necrotic tissue is usually evident (Fig. 18) (Galeotti et al. 1995, 1996, Essam et al. 2016).

The **chronic form** of the disease is generally observed in adult fish, as a result of previous acute infections that have been overcome. The only lesions are observed in the spleen, which is increased in volume and scattered with point-like whitish nodular formations ranging in size from a grain of millet to a few millimetres, sometimes confluent, often protruding on the surface (Fig. 21), which involve alteration of the organ architecture (Fig. 22). On a histological level, it is possible to observe that these lesions evolve from simple necrotic foci to true granulomas, characterized by a necrotic centre

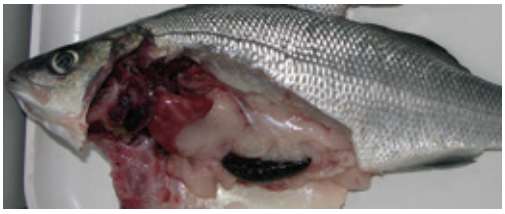

Figure 15. Juvenile sea bass affected by acute photobacteriosis caused by Phdp: slight diffuse hemorrhages are evident in the abdominal cavity and the spleen appears splenomegalic with scattered with small whitish areas.

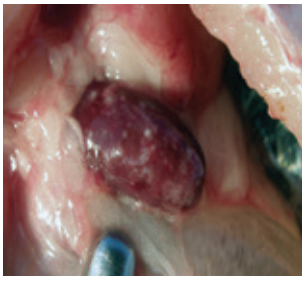

Figure 16. Splenomegalic spleen at greater magnification in juvenile bass affected by acute photobacteriosis caused by Phdp: small whitish punctate lesions caused by necrotic foci are visible.

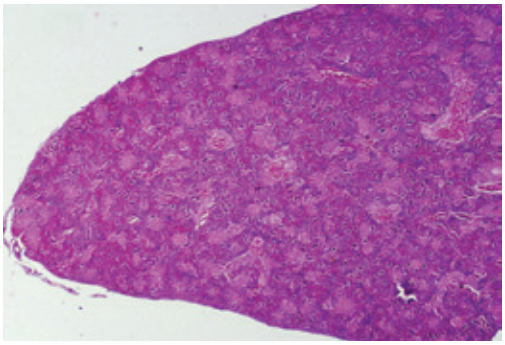

Figure 17. Histological picture of the spleen in juvenile sea bass affected by acute photobacteriosis caused by Phdp: the presence of necrotic foci with evident state of reactivity and presence of numerous bacterial colonies. H&H.

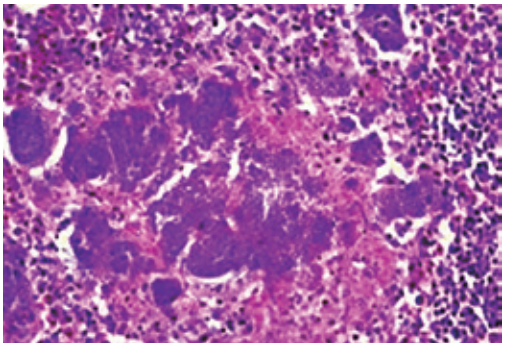

Figure 18. Detail of Fig. 17: bacterial colonies and macrophages are visible, surrounded by abundant acidophilic necrotic tissue. H&H.

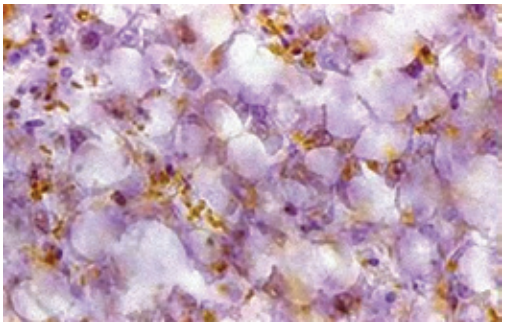

Figure 19. *Photobacterium damselae* subsp. *piscicida* labelled with specific antibody in sea bass liver. IHC.

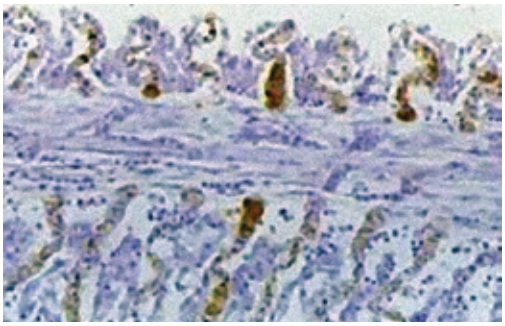

Figure 20. Presence of bacterial emboli and macrophages loaded with bacteria, labelled with specific antibodies within the capillaries of the primary gill lamellae. IHC.

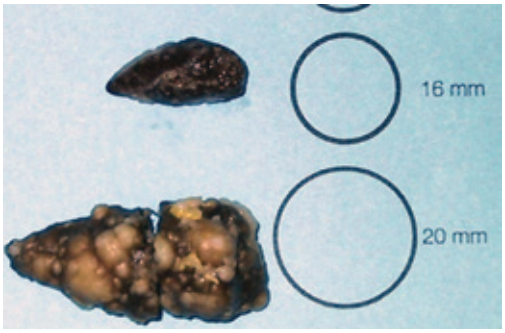

Figure 21. Spleen in adult sea bass affected by chronic photobacteriosis: the spleen is enlarged and covered with nodular formations, whitish from a grain of millet to a few millimetres in size, sometimes confluent, often protruding on the surface.

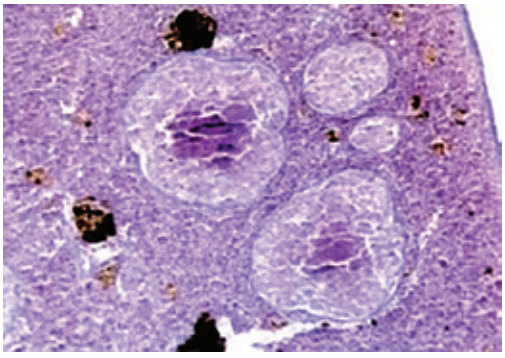

Figure 22. Histological preparation referred to Fig. 21: granulomas formed by a necrotic centre surrounded by macrophages, epithelioid cells and fibroconnectival reaction are observed. Van Gieson Weigert trichrome.

where no bacteria are found (negative IHC with specific antibody), surrounded by macrophages, epithelioid cells and a fibroconnective reaction. These granulomas resemble in appearance the typical nodules that are observed in the spleen of fish with mycobacteriosis and this is the reason why photobacteriosis has been called pseudotuberculosis for years (Galeotti et al. 1995, 1996).

3.3.4. Disease management

Vaccination is the ideal prophylactic measure for the control of infectious diseases in fish, and numerous researches have been dedicated to the development of effective vaccines against Phdp for use in different farmed fish species (Micoli et al. 2019, 2021). Conventional formulations consisting of heat or formalin-inactivated bacterial cells (bacterin) have shown a variable efficacy when administered by immersion, intraperitoneal injection (IP) or oral route, in both monovalent and bivalent forms (consisting of Phdp bacterin and *V. alginolyticus* or *V. anguillarum* bacterin) (Magariños et al. 1994b, 1999, Moriñigo et al. 2002, Paolini et al. 2005, Madonia et al. 2017). The use of vaccines consisting of bacterin seems ideal for the vaccination of larvae at 90 dph, fry and juveniles but also breeding stocks, as it would allow a certain degree of immunity to be transferred to the larvae as evidenced in sea bream (Hanif et al. 2004). Vaccines consisting of attenuated bacteria or extracellular products (ECPs) administered by immersion are more protective, as they induce the activation of a more intense antibody (IgM) response in the gill and intestinal mucosae as demonstrated in sea bass (Dos Santos et al. 2001). Similarly, the immersion administration of a mixture of inactivated cells and ECPs induced a good level of protection in sea bass by stimulating the synthesis of specific IgM (Bakopoulos et al. 2003). On the other hand, the use of adjuvants is useful in enhancing the effectiveness of vaccine formulations against Phdp as they stimulate a greater synthesis of specific antibodies than the non-adjuvanted ones (Micoli et al. 2021). Approaches based on recent biomolecular and DNA recombination techniques have been used to a very limited extent for the development of bacterial vaccines for fish and effective formulations against photobacteriosis are not yet available (Micoli et al. 2019, 2021).

In addition to vaccination, the implementation of adequate sanitary and biosecurity measures in fish farms, the reduction of stress (reduction of handling) and the immunostimulation through the dietary administration of probiotics or algae demonstrated effectiveness in the disease prevention (Couso et al. 2003, Peixoto et al. 2019, Abdala-Díaz et al. 2021, Gutiérrez Falcón et al. 2021). Furthermore, the selection of fish breeding stocks that are genetically resistant to photobacteriosis constitutes a potential strategy to be pursued to reduce the likelihood of this disease on farms and avoid economic losses (Micoli et al. 2019).

When the prophylaxis is not adequately applied, an early diagnosis is essential to control photobacteriosis outbreaks and the application of antimicrobials is inevitable.

Generally, prompt treatment of infected fish with sulfa-trimethoprim and flumequine is effective in limiting the spread of the pathogen. Instead, the use of other antibiotics such as tetracyclines, sulfonamide, ampicillin, chloramphenicol, florfenicol and erythromycin have shown moderate/weak efficacy and genes encoding for resistance factors have been documented in various Phdp strains (Andreoni and Magnani 2014, Essam et al. 2016). Recently, the antibacterial activity of extracts obtained from medicinal plants against Phdp has been highlighted (Bulfon et al. 2014) and the use of other natural substances such as marine natural products (MNPs) or antimicrobial peptides (AMPs) is currently in an experimental phase and constitutes a future possible alternative to conventional antibiotics.

References

Abdala-Díaz, R. T., García-Márquez, J., Rico, R. M., Gómez-Pinchetti, J. L., Mancera, J. M., Figueroa, F. L., & Moriñigo, M. Á. 2021. Effects of a short pulse administration of Ulva rigida on innate immune response and intestinal microbiota in *Sparus aurata* juveniles. *Aquaculture Research*, 52, 3038-3051.

Acosta, F., Vivas, J., Padilla, D., Vega, J., Bravo, J., Grasso, V., & Real, F. 2009. Invasion and survival of Photobacterium damselae subsp. piscicida in non-phagocytic cells of gilthead sea bream, *Sparus aurata* L. *Journal of Fish Diseases*, 32, 535-541.

Andreoni, F., & Magnani, M. 2014. Photobacteriosis: prevention and diagnosis. *Journal of immunology Research*, *2014*.

Bakopoulos, V., Hanif, A., Poulos, K., Galeotti, M., Adams, A., & Dimitriadis, G. J. 2004. The effect of in vivo growth on the cellular and extracellular components of the marine bacterial pathogen *Photobacterium damsela* subsp. *piscicida*. *Journal of Fish Diseases*, 27, 1-13.

Bakopoulos, V., Poulos, K., Adams, A., Galeotti, M., & Dimitriadis, G. J. 2002. The effect of novel growth media on the virulence and toxicity of cellular and extracellular components of the fish pathogen *Photobacterium damselae* subsp. *piscicida*. *Bulletin of the European Association of Fish Pathologists*, 22, 272-279.

Bakopoulos, V., Volpatti, D., Gusmani, L., Galeotti, M., Adams, A., & Dimitriadis, G. J. 2003. Vaccination trials of sea bass, *Dicentrarchus labrax* (L.), against *Photobacterium damsela* subsp. *piscicida*, using novel vaccine mixtures. *Journal of Fish Diseases*, 26, 77-90.

Barnes, A. C., & Ellis, A. E. 2004. Bacterial Diseases of Fish—Where Do We Go from Here?. *Current trends in the study of bacterial and viral fish and shrimp diseases*, 1-38.

Bulfon, C., Volpatti, D., & Galeotti, M. 2014. In vitro antibacterial activity of plant ethanolic extracts against fish pathogens. *Journal of the world aquaculture society*, 45, 545-557.

Couso, N., Castro, R., Magariños, B., Obach, A., & Lamas, J. 2003. Effect of oral administration of glucans on the resistance of gilthead seabream to pasteurellosis. *Aquaculture*, 219, 99-109.

Dos Santos, N. M., Taverne-Thiele, J. J., Barnes, A. C., van Muiswinkel, W. B., Ellis, A. E., & Rombout, J. H. 2001. The gill is a major organ for antibody secreting cell production following direct immersion of sea bass (*Dicentrarchus labrax*, L.) in a *Photobacterium damselae* ssp. *piscicida* bacterin: an ontogenetic study. *Fish & shellfish immunology*, 11, 65-74.

do Vale, A., Silva, M. T., dos Santos, N. M., Nascimento, D. S., Reis-Rodrigues, P., Costa-Ramos, C., ... & Azevedo, J. E. 2005. AIP56, a novel plasmid-encoded virulence factor of *Photobacterium damselae* subsp. *piscicida* with apoptogenic activity against sea bass macrophages and neutrophils. *Molecular microbiology*, 58, 1025-1038.

Essam, H. M., Abdellrazeq, G. S., Tayel, S. I., Torky, H. A., & Fadel, A. H. 2016. Pathogenesis of *Photobacterium damselae* subspecies infections in sea bass and sea bream. *Microbial pathogenesis*, 99, 41-50.

Galeotti, M., Bakopoulos, V., D'Angelo, L., Manetti, M.F., Musetti, M., Volpelli, L., Volpatti, D., Adams, A. 1996. Histological, immunohistochemical and ultrastructural examination of pasteurellosis experimentally induced in sea bass (*Dicentrarchus labrax*). In *14th Congress of the European Society of Veterinary Pathology, Ghent*, 162.

Galeotti, M., Volpelli, L., Volpatti, D., & Del Grano, N. 1995. Pasteurellosis experimentally induced in sea bass (*Dicentrarchus labrax*): histological and immunohistochemical evaluation. In *17th Congress of the European Society of Veterinary Pathology, Palma De Mallorca*, 51.

Gauthier, G., Lafay, B., Ruimy, R., Breittmayer, V., Nicolas, J. L., Gauthier, M., & Christen, R. 1995. Small-Subunit rRNA Sequences and Whole DNA Relatedness Concur for the Reassignment of *Pasteurella piscicida* (Snieszko et al.) Janssen and Surgalla to the Genus *Photobacterium* as *Photobacterium damsela* subsp. *piscicida* comb. nov. *International Journal of Systematic and Evolutionary Microbiology*, 45, 139-144.

Gutiérrez Falcón, A., Padilla, D., Real, F., Ramos Sosa, M. J., Acosta-Hernández, B., Sánchez Henao, A., ... & Martín-Barrasa, J. L. 2021. Screening of New Potential Probiotics Strains against *Photobacterium damselae* subsp. *piscicida* for Marine Aquaculture. *Animals*, 11, 2029.

Hanif, A., Bakopoulos, V., Leonardos, I., & Dimitriadis, G. J. 2005. The effect of sea bream (*Sparus aurata*) broodstock and larval vaccination on the susceptibility by *Photobacterium damsela* subsp. *piscicida* and on the humoral immune parameters. *Fish & shellfish immunology*, 19, 345-361.

Jung, T. S., Thompson, K. D., Volpatti, D., Galeotti, M., & Adams, A. 2007. Variation in the molecular weight of *Photobacterium damselae* subsp. *piscicida* antigens when cultured under different conditions in vitro. *Journal of Veterinary Science*, 8, 255-261.

Jung, T. S., Thompson, K. D., Volpatti, D., Galeotti, M., & Adams, A. 2008. In vivo morphological and antigenic characteristics of *Photobacterium damselae* subsp. *piscicida*. *Journal of Veterinary Science*, 9, 169-175.

Madonia, A., Melchiorri, C., Bonamano, S., Marcelli, M., Bulfon, C., Castiglione, F., ... & Romano, N. 2017. Computational modeling of immune system of the fish for a more effective vaccination in aquaculture. *Bioinformatics*, 33, 3065-3071.

Magariños, B., Romalde, J.L., Barja, J.L., Núñez, S., & Toranzo, A.E. 1999. Protection of gilthead seabream against pasteurellosis at the larval stages. *Bulletin of European Association of Fish Pathologists*, 19, 159–161.

Magariños, B., Bonet, R., Romalde, J. L., Martınez, M. J., Congregado, F., & Toranzo, A. E. 1996a. Influence of the capsular layer on the virulence of *Pasteurella piscicida* for fish. *Microbial Pathogenesis*, 21, 289-297.

Magariños, B., Romalde, J. L., Noya, M., Barja, J. L., & Toranzo, A. E. 1996b. Adherence and invasive capacities of the fish pathogen *Pasteurella piscicida*. *FEMS Microbiology Letters*, 138, 29-34.

Magariños, B., Romalde, J. L., Lemos, M. L., Barja, J. L., & Toranzo, A. E. 1994a. Iron uptake by *Pasteurella piscicida* and its role in pathogenicity for fish. *Applied and Environmental Microbiology*, 60, 2990-2998.

Magariños, B., Romalde, J. L., Santos, Y., Casal, J. F., Barja, J. L., & Toranzo, A. E. 1994b. Vaccination trials on gilthead seabream (*Sparus aurata*) against *Pasteurella piscicida*. *Aquaculture*, 120, 201-208.

Miccoli, A., Manni, M., Picchietti, S., & Scapigliati, G. 2021. State-of-the-art vaccine research for aquaculture use: The case of three economically relevant fish species. *Vaccines*, 9, 140.

Miccoli, A., Saraceni, P. R., & Scapigliati, G. 2019. Vaccines and immune protection of principal Mediterranean marine fish species. *Fish & shellfish immunology*, 94, 800-809.

Moriñigo, M. A., Romalde, J. L., Chabrillon, M., Magariños, B., Arijo, S., Balebona, M. C., & Toranzo, A. E. 2002. Effectiveness of a divalent vaccine for gilt-head sea bream (*Sparus aurata*) against *Vibrio alginolyticus* and *Photobacterium damselae* subsp. *piscicida*. *Bulletin-European Association of Fish Pathologists*, 22, 298-303.

Paolini, A., Ridolfi, V., Zezza, D., Cocchietto, M., Musa, M., Pavone, A., ... & Giorgetti, G. 2005. Vaccination trials of sea bass (*Dicentrarchus labrax*) against pasteurellosis using oral, intraperitoneal and immersion methods. *Veterinaria Italiana*, 41, 137-144.

Pellizzari, C., Krasnov, A., Afanasyev, S., Vitulo, N., Franch, R., Pegolo, S., ... & Bargelloni, L. 2013. High mortality of juvenile gilthead sea bream (*Sparus aurata*) from photobacteriosis is associated with alternative macrophage activation and anti-inflammatory response: results of gene expression profiling of early responses in the head kidney. *Fish & shellfish immunology*, 34, 1269-1278.

Peixoto, M. J., Ferraz, R., Magnoni, L. J., Pereira, R., Gonçalves, J. F., Calduch-Giner, J., ... & Ozório, R. O. 2019. Protective effects of seaweed supplemented diet on antioxidant and immune responses in European seabass (*Dicentrarchus labrax*) subjected to bacterial infection. *Scientific reports*, 9, 1-12.

Romalde, J. L. 2002. *Photobacterium damselae* subsp. *piscicida*: an integrated view of a bacterial fish pathogen. *International Microbiology*, 5, 3-9.

Snieszko, S. F., Bullock, G. L., Hollis, E., & Boone, J. G. 1964. *Pasteurella* sp. from an epizootic of white perch (*Roccus americanus*) in Chesapeake Bay tidewater areas. *Journal of Bacteriology*, 88, 1814-1815.

3.4. Tenacibaculosis caused by *Tenacibaculum maritimum*

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3.4.1. Pathogen

Tenacibaculum maritimum is the etiological agent of an ulcerative disease known as tenacibaculosis. The disease affects numerous marine fish species throughout the whole world (Toranzo et al. 2015), causing considerable economic impact due to high mortality rates, increased susceptibility to other infections and enormous costs for treatment of farmed fish (Avendano-Herera et al. 2006).

The disease was reported for the first time in Japan in 1977 as the cause of massive mortalities in *Pagrus major* and *Acanthopagrus schlegeli* in a hatchery (Wakabayashi et al. 1986). The bacterium recovered from the affected fish was identified as *Flexibacter maritimus*, previously known as *Cytophaga marina.* Phenotypic, chemotaxonomic and phylogenetic characteristics were then analyzed using both 16S rRNA and GyrB sequences, therefore the bacterium was reclassified to the new genus *Tenacibaculum* and it was denominated *Tenacibaculum maritimum* (Suzuki et al. 2001).

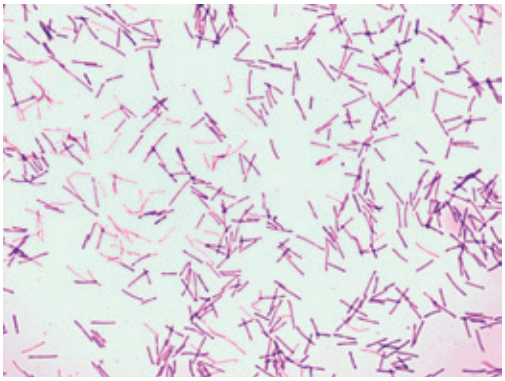

Figure 23. Filamentous *T. maritimum* in Gram-stained smear

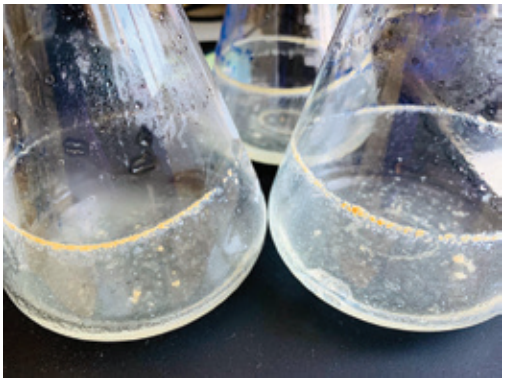

Figure 24. Biofilm formation on the walls of the cultivation vessel

T. maritimum is a gram-negative filamentous bacterium, 2-30 µm long and 0.5 µm in diameter (Fig. 23), showing gliding motility on wet surfaces (Fig. 24) (Wakabayashi et al. 1986). Colonies are flat, pale yellow with irregular margins and adhere strongly to *Flexibacter maritimum* medium (FMM), while bacterial cells do not contain a cell-wall-associated flexirubin–type pigment (Avendano-Herera et al. 2004).

3.4.2. Infection and ecological factors

The strong adherence to the skin mucus and the capacity to resist its bactericidal activity are pointed out as possible virulence factors of *T. maritimum* (Margarinos et al. 1995). Moreover, the extracellular products (ECP) released by this bacterium possess very high proteolytic activity, which increases its pathogenicity.

The disease outbreaks are influenced by a multiplicity of ecological factors like fish stress and immunosuppression, water salinity variation, UV light, lack of sand substrate in the tank, fish high density and poor feeding. The disease severity and ratio of prevalence seem to be dependent on a high water temperature (above 15°C), a salinity ranging from 30 to 35‰ and low water quality (Avendano-Herera et al. 2006). No host specificity has been documented and wild fish may serve as reservoirs of infection since some studies allude to the involvement of jellyfish and sea lice as vectors of *T. maritimum* (Ferguson et al. 2010). The chronic presence of the bacterium in the mucus layer suggests that also fish mucus could be a reservoir of infection (Avendano-Herrera et al. 2005).

3.4.3. Clinical aspects of the disease

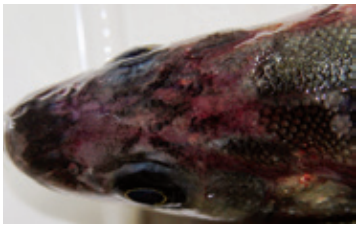

Figure 25. Typical lesions on the head of the seabass affected by tenacibaculosis

Figure 26. Typical lesions on the jaws, opercula and body of sea bass affected by tenacibaculosis

Figure 27. Yellow mats on the pale gills of sea bass affected by tenacibaculosis

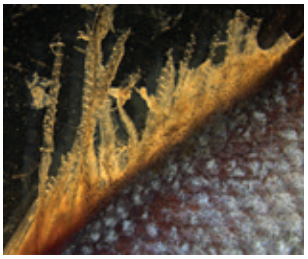

Figure 28. "Frayed fins" of sea bass affected by tenacibaculosis

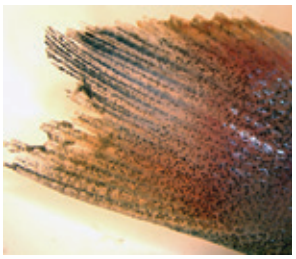

Figure 29. "Tail rot" of sea bass affected by tenacibaculosis

T. maritimum is an opportunistic pathogen that primarily causes extensive skin lesions and gill abrasion, and subsequently systemic infections. Affected fish show loss of

appetite, become lethargic and show skin lesions around the eyes and on the head (Fig. 25-29). The lesions are characterized by increased mucus production and the presence of whitish necrotic tissue (Smage et al. 2016). Fish with gill infections have increased respiratory rate with visible yellow or brown mats on the pale gills, and extensive areas of severe necrosis (Mitchell & Rodger 2011).

Fish of all ages may be infected by *T. maritimum* but younger fish suffer a more severe form of the disease (Tobar 2015). Bacterium poses a high adaptation capacity and therefore the disease may occur at different temperatures, but stress, salinity variation and immunosuppression are triggers for disease outbreaks.

Based on the above described clinical aspects, the disease is also known as ”frayed fins and tail rots”, “necrosis of the gills and eyes”, “gliding bacterial disease of sea fish” and “eroded mouth syndrome” (Toranzo et al. 2005).

3.4.4 Disease management

A single commercial vaccine against *T. maritimum* has been developed for the injection vaccination of turbot (*Scophthalmus maximus*) in Spain, inducing a satisfactory relative per cent survival (RPS), three months after vaccination and giving protection up to 6 months (Romalde et al. 2005). Reports on experimental vaccination using autologous vaccines showed promising results. Currently, the vaccination strategy used in the management of tenacibaculosis in Chile is based on an intraperitoneal priming and a subsequent oral booster to prolong the duration of immunity and prevent the inflammatory reaction caused by the vaccine injection.

Still, the disease outbreaks should be prevented by implementing GAP such as control of fish density, reducing stress conditions, avoiding fish overfeeding and skin damage due to manipulation. In some cases, the antimicrobial treatment of fish is inevitable but an antibiogram to test the *in vitro* antibiotic susceptibility of isolated bacteria should be applied. A study of different *T. maritimum* isolates from different geographical areas showed a similar pattern of susceptibility to tetracycline, potentiated sulphonamides and fluoroquinolones while resistance to kanamycin, neomycin, and quinolones.

References

Avendaño-Herrera, R., Toranzo, A. E., & Magariños, B. 2006. Tenacibaculosis infection in marine fish caused by *Tenacibaculum maritimum*: a review. *Diseases of aquatic organisms*, 71, 255-266.

Avendaño-Herrera, R., Magariños, B., López-Romalde, S., Romalde, J. L., & Toranzo, A. E. 2004. Phenotypic characterization and description of two major O-serotypes in *Tenacibaculum maritimum* strains from marine fishes. *Diseases of aquatic organisms*, 58, 1-8.

Ferguson, H. W., Christian, M. D., Hay, S., Nicolson, J., Sutherland, D., & Crumlish, M. 2010. Jellyfish as vectors of bacterial disease for farmed salmon (*Salmo salar*). *Journal of veterinary diagnostic investigation*, 22, 376-382.

Magarinos, B., Pazos, F., Santos, Y., Romalde, J. L., & Toranzo, A. E. 1995. Response of *Pasteurella piscicida* and *Flexibacter maritimus* to skin mucus of marine fish. *Diseases of Aquatic Organisms*, 21, 103-108.

Mitchell, S. O., & Rodger, H. D. 2011. A review of infectious gill disease in marine salmonid fish. *Journal of fish diseases*, 34, 411-432.

Romalde, J. L., Ravelo, C., López-Romalde, S., Avendano-Herrera, R., Magariños, B., & Toranzo, A. E. 2005. Vaccination strategies to prevent emerging diseases for Spanish aquaculture. *Developments in biologicals*, 121, 85-95.

Småge, S. B., Frisch, K., Brevik, Ø. J., Watanabe, K., & Nylund, A. 2016. First isolation, identification and characterisation of *Tenacibaculum maritimum* in Norway, isolated from diseased farmed sea lice cleaner fish *Cyclopterus lumpus* L. *Aquaculture*, 464, 178-184.

Suzuki, M., Nakagawa, Y., Harayama, S., & Yamamoto, S. 2001. Phylogenetic analysis and taxonomic study of marine Cytophaga-like bacteria: proposal for *Tenacibaculum* gen. nov. with *Tenacibaculum maritimum* comb. nov. and *Tenacibaculum ovolyticum* comb. nov., and description of *Tenacibaculum mesophilum* sp. nov. and *Tenacibaculum amylolyticum* sp. nov. *International journal of systematic and evolutionary microbiology*, 51, 1639-1652.

Tobar, J. 2015. Current situation of *Tenacibaculum* in Chile and potential prevention strategies. In *Tenacibaculum maritimum*: Current Knowledge and Future Directions. Workshop Report and Synopsis, Campbell River: CAHS workshop. pp. 11

Toranzo, A.E. 2015. Tenacibaculosis of farmed fish in Southern Europe. In: *Tenacibaculum maritimum*: Current Knowledge and Future Directions. Workshop Report and Synopsis, Campbell River: CAHS workshop. pp. 8

Toranzo, A. E., Magariños, B. & Romalde, J. L. 2005. A review of the main bacterial fish diseases in mariculture systems. *Aquaculture*, 246, 37-61.

Wakabayashi, H., Hikida, M. & Masumura, K. 1986. *Flexibacter maritimus* sp. nov., a pathogen of marine fishes. *International Journal of Systematic and Evolutionary Microbiology*, 36, 396-398.

4. VACCINATION AND VACCINATION STRATEGY

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4.1. Introduction

Vaccination is a key component of sustainable and healthy farmed fish production (Miccoli et al. 2019) and it has been recognised as an essential prophylactic measure to reduce the use of antibiotics within the aquaculture industry (Adams 2019).

From the economic aspect, vaccination is the best method to increase the fish survival rate and profitability of aquaculture, in combination with other factors necessary to guarantee the achievement of the highest possible fish growth performance such as high-quality fingerlings, good nutrition, good farming and husbandry practices, and health management (McLoughlin 2016). Vaccination allows to control of the spread of infectious diseases on farms, saves costs, and reduces the need for antibiotics and chemicals avoiding consequently the problems related to antibiotic resistance, and concern over the deleterious impact of residues in the environment (Adams, 2016).

Vaccination aims to induce a long term immunity against one or more pathogens as it stimulates the fish-specific immune responses and protects them against one or more specific infectious diseases. A good vaccine should fulfil several requirements: i) it should be safe and non-toxic, ii) it should be cost-effective, iii) it should secure long-term protection, iv) it should protect the fish in the age or before the period when they are most susceptible to the diseases, v) it should be administered to fish by a route that should be adjusted based on the fish age and type of vaccine.

4.1.1. Types of vaccines

There are different types of vaccines (Ma et al. 2019):

1) inactivated whole-cell vaccines are vaccines prepared with the pathogen(s) inactivated using different methods and they may be supplemented with adjuvants
2) live attenuated vaccines are modified vaccines prepared from a live pathogen(s) displaying attenuated virulence or natural low virulence toward the target fish species or they may be attenuated by physical or chemical procedures, culturing under abnormal conditions, or genetic manipulation
3) subunit vaccines are vaccines designed by using only antigenic components for vac-

cination and that cannot replicate in the host, therefore there is no risk of pathogenicity to the host or non-target species (Hanson et al. 2000)

4) recombinant vaccines are produced through recombinant DNA technology that involves the insertion of DNA encoding for an antigen, which will be expressed in bacterial or mammalian cells, then purified and used for immunization (Lorenzen 1999)
5) nucleic acid vaccines (DNA or RNA) consist of DNA or RNA encoding for the antigen(s) of interest; they are relatively simple to generate and safe to administer since they cannot revert to a pathogenic state (Ulmer et al. 2012)
6) synthetic vaccines are composed mainly or wholly of synthetic peptides, carbohydrates or antigens (Adams 2019).

Currently, most of the commercially available vaccines are prepared with the whole pathogen(s) inactivated using heat or formaldehyde. In the case of bacteria, they contain pathogenic bacterial cells and their extracellular products which are part of the infective mechanism. Bacteria are cultured on a medium and then deactivated. These vaccines could be adjuvanted or used without adjuvant: for example, the vaccines prepared for intraperitoneal application are mostly adjuvanted (Ribeiro 2010).

Adjuvants are pharmacological or immunological agents capable of modifying the effect of other agents, such as drugs or vaccines. When given together with a vaccine, the adjuvant will induce a more effective stimulation of the immune response of the individual that was submitted to vaccination and increase its response to the vaccine. Aluminium salt, virosomes and certain oils are all commonly used as adjuvants for fish vaccines.

Vaccines may be monovalent or polyvalent. Monovalent vaccines are capable to protect fish from one microorganism whereas the polyvalent vaccine may immunize fish against two or more different microorganisms. Generally, it seems that monovalent vaccines are developing a stronger immune response.

4.1.2. Vaccine administration

Three different methods of vaccine administration are used in fish:

1) injection vaccination - may be intraperitoneal or intramuscular: this method of vaccination is time-consuming, is labour intensive requires additional equipment and skilful operators, and the time needed to vaccinate one cage is longer compared to immersion vaccination; during vaccine injection, fish should be anaesthetized and of an appropriate size; however, the duration of the induced immunity is longer compared to the immunity obtained after immersion vaccination;
2) immersion or dip vaccination is an appropriate method for hatcheries and small-sized fish: this method implies dipping of fish batch during a specific time into a mixture of vaccine and farming water; the advantage is that a large number of fish can

be vaccinated at the same time, does not require additional equipment than those usually used at the farm but the quantities of vaccine needed for the immunization of one cage of fish are higher than those needed for an injection vaccination and the induced immunity lasts shorter compared to the injection vaccination;

3) oral vaccination consists of the delivery of vaccine through the feed: it is the simplest method of vaccination, not harmful for vaccinated fish and it does not require any equipment or special skill of the operators; the main bottlenecks of this method are that it provokes neither strong nor long-lasting immunity, it is necessary to give a higher dose of antigen compared to injection and immersion vaccination to obtain an effective immunity in fish; the poor immune response developed is due to low pH and high enzymatic activity in the foregut which destroys the vaccine and the vaccine microencapsulation can overpass these obstacles, but still the immune response of the vaccinated fish is not so good as after the vaccine administration by injection.

The optimal effects of vaccines do not depend solely on the choice of the vaccine and vaccine administration but also on the species to be vaccinated, the status of the fish immune system, production cycle and life history, what diseases we want to control when these diseases occur, farming technology, environmental conditions (temperature, salinity), stress factors, nutrition and cost-benefit study.

The awareness that vaccines will not be effective for ongoing disease outbreaks but will be used to prevent a specific disease outbreak should be raised. Also, farmers should be aware that vaccination can protect fish from the outbreak of an infectious disease but it does not protect fish if performed when a disease outbreak is already detected on the farm.

References

Adams, A. 2019. Progress, challenges and opportunities in fish vaccine development. *Fish & shellfish immunology*, 90, 210-214.

Hansson, M., Nygren, P. A. K., & Stahl, S. 2000. Design and production of recombinant subunit vaccines. *Biotechnology and applied biochemistry*, 32, 95-107.

Lorenzen N. 1999. Recombinant vaccines: experimental and applied aspects. Fish & Shellfish Immunology, 9, 361-365

Ma, J., & Bruce, J. T., M. Jones, E. y D. Cain, K. A. 2019. Review of Fish Vaccine Development Strategies: Conventional Methods and Modern Biotechnological Approaches. *Microorganisms*, 7, 569.. doi:10.3390/microorganisms7110569.

McLoughlin, M. 2016. Fish Vaccination - A brief overview, Aquatic Veterinary Services Belfast. https://kipdf.com/fish-vaccination-a-brief-overview-dr-marian-mcloughlin-aquatic-veterinary-servic_5ad31cda7f8b9aa8988b459a.html (accessed online 5th February 2022)

Miccoli, A., Saraceni, P. R., & Scapigliati, G. 2019. Vaccines and immune protection of principal Mediterranean marine fish species. *Fish & shellfish immunology*, 94, 800-809.

Ribeiro, C., & Schijns, V. E. 2010. Immunology of vaccine adjuvants. In: *Vaccine adjuvants* (pp. 1-14). Humana Press, Totowa, NJ.

Ulmer, J. B., Mason, P. W., Geall, A., & Mandl, C. W. 2012. RNA-based vaccines. *Vaccine*, 30, 4414-4418.

4.2. List of available commercial vaccines

Table 2. Commercial vaccines for use in farming of European sea bass and gilthead seabream

Product name	Producer	Active substance	Mode of use	Dose	Registered		
					Croatia	Italy	Centrally EU
AlphaDip Vib	Pharmaq Zoetis	Inactivated *Vibrio anguillarum*	Dip*	1:20	yes	yes	no
AlphaJect 2000	Pharmaq Zoetis	Inactivated *V.anguillarum*, *Photobacterium damselae* subsp. *piscicida*	Injection	0.1 mL	yes	no	yes
AlphaJect micro 2000	Pharmaq Zoetis	Inactivated *V.anguillarum*, *Photobacterium damselae* subsp. *piscicida*	Injection	0.05 mL	yes	yes	yes
AlphaJect micro Noda	Pharmaq Zoetis	Inactivated NNV genotype RGNNV	Injection	0.05 mL	yes	yes	yes
Icthiovac VNN	LABORATORIS HIPRA	Inactivated noda virus	Injection	0.1 mL	yes	yes	yes
Ichtiovac VR	LABORATORIS HIPRA	Inactivated *Vibrio anguillarum*	Bath* 10s Bath* 1 h	1:10 1:500	ne	yes	yes
Ichtiovac VR/PD	LABORATORIS HIPRA	Inactivated *V.anguillarum*, *Ph.damselae* subsp. *piscicida*	Injection	0.1 mL	yes	yes	yes
Ichtiovac PD	LABORATORIS HIPRA	Inactivated *Ph.damselae* subsp. *piscicida*	Bath* 10s Bath* 1 h	1:10 1:500	yes	yes	yes
MARIMARK	Benchmark Animal Health	Inactivated NNV genotype RGNNV	Injection	0.1 mL	yes	no	no
VIBRIFISH VAX	FATRO	Inactivated *Vibrio anguillarum*	Immersion*	1:10	no	yes	no
VIBRIFISH VAX	FATRO	Inactivated *Vibrio anguillarum*	Injection	0.1 mL	no	yes	no
	MSD ANIMAL HEALTH	Inactivated *Vibrio anguillarum*	Immersion*	1:10	no	yes	yes

*Different producers are addressing dip, immersion or bath as a mode of vaccine administration Usually, we use immersion as a generic term, dip for a quick immersion and bath for a longer one. Therefore, the instructions for use should be carefully studied and followed before use.

Source:

Ministry of agriculture. Veterinary and Food Safety Directorate. 2022. http://veterinarstvo.hr/default.aspx?id=140. Accessed January 21 2022

Ministero della Salute. Prontuario dei medicinali veterinary versione 1.2.0. 2022. https://www.vetinfo.it/j6_prontuario/public/

https://www.ema.europa.eu

4.3. Vaccination in hatchery

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It is known that newly hatched marine fish are unable to synthesize antibodies until several weeks after hatching but they are provided by innate immunity with wide capacity and mechanisms of protection against pathogens (Galindo-Villegas & Mulero 2014).

In the hatchery of European seabass and gilthead seabream, the first vaccination is usually applied when they reach a weight of 1 g or more. At that size, the vaccines are administered by bath and the induced immunity lasts no longer than 4 to 6 months due to incomplete development of the immune system. For this reason, the re-vaccination of larger fish is required before the translocation in cages; in this case, the vaccines are most often administered by intraperitoneal injections.

In this chapter, we'll try to point out all the necessary steps needed to perform successful vaccination in the hatchery of sea bass and sea bream.

One of the key information to keep in mind is that only healthy fish will develop an optimal immunity after vaccination.

A. Week before the planned vaccination some important points should be addressed:

1. a thorough health check including fish behaviour, appetite and mortality should be performed before planned vaccination
2. if any disease is diagnosed, fish should be appropriately treated before the planned vaccination
3. a diet with commercially available immunostimulants could be administered to fish for one or two weeks before the vaccination, to improve the fish immune response induced by the vaccine and the outcome of vaccination treatment (it is not compulsory)
4. fish should be of suitable size for vaccination
5. the amount of fish for vaccination should be verified and a sufficient quantity of vaccine should be available and correctly stored at 2 – 8 °C (it should not be frozen and should be protected from sunlight)

6. the quality and expiry date of the vaccine should be checked

B. One or two days before vaccination additional points should be addressed:

1. all equipment used for vaccination or that could come in contact with fish should be thoroughly washed and disinfected
2. tanks in which fish will be placed after vaccination should also be cleaned and disinfected
3. a vaccination plan with trained personnel should be set up so that each person should know what he will work and when
4. the average weight of the fish batch to be vaccinated should be determined (recommended size of fish is 1 to 5 grams)
5. fish should starve a day before vaccination

C. Vaccination procedure

1. remove the bottle of vaccine from the refrigerator and leave it to acquire the environmental temperature, shake it well to be sure that the content is evenly mixed
2. dilute the content immediately after opening with hatchery water according to the instruction for use issued by the vaccine producer and mix it thoroughly
3. avoid the mixing of vaccines with other products
4. vaccinate fish in batches, weight vaccinated fish to be aware of the fish quantity and volume of vaccinated fish (Fig. 30)

Figure 30. Recommendable batches of fish to be submitted for vaccination

Figure 31. Immersion of fry into vaccine

Figure 32. Vaccination in the hatchery

5. keep fry immersed in the vaccine according to the instructions of the vaccine producer (30 to 60 seconds or even more for some vaccines) (Fig. 31, 32)

6. try to prevent a further dilution of the vaccine with water from hatchery tanks when moving the fish to the vaccination vessel
7. move the vaccinated fish batch immediately into a clean new tank
8. ensure that personnel involved in vaccination wear suitable protection such as rubber gloves and protective glasses
9. discard any used vaccine containers and the waste vaccine according to the manufacturer's instructions
10. big hatcheries may use the machine for the immersion vaccination of fish (Fig. 33, 34)

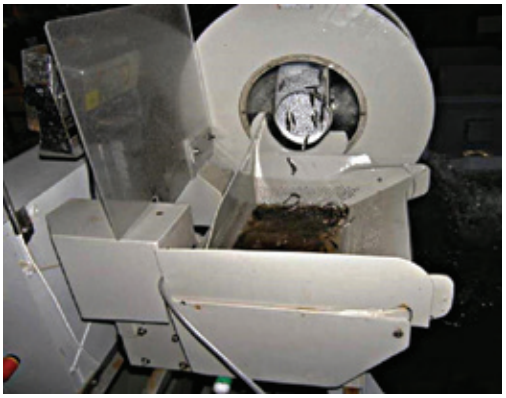

Figure 33 & 34. Machine vaccination of fry in big hatchery

D. After vaccination

1. carefully observe the behaviour of vaccinated fish and slowly start to feed them again the next day after immunization
2. immunity starts to develop after 2 weeks if fish are maintained at 21±2 ^{o}C and it lasts about 10 weeks at the same temperature
3. fish should be vaccinated at least 2 weeks before removing from hatchery/nursery to cages

References

Galindo-Villegas, J., & Mulero, V. 2015. Current knowledge on the development and functionality of immune responses in the European sea bass (*Dicentrarchus labrax). Biology of European sea bass*, 342-373.

RUMA (Responsible Use of Medicines in Aquaculture Alliance) 2006. Responsible use of vaccines and vaccination in fish production. Available at: www.ruma.org.uk

4.4. Revaccination in the cages

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Immunity obtained by vaccination performed when fish are in hatchery lasts about 10 weeks, therefore revaccination of fry or juveniles is inevitable to also protect them during the cage farming when they will be more susceptible to the infectious diseases described in the first chapter of this document and to avoid significant economic losses. The losses of fish could be mitigated by the administration of antibacterial drugs, but very often the disease is not diagnosed during the prodromic period (the stage when fish are infected but do not show clinical symptoms yet), hence the efficacy of the pharmaceutical treatment could be questionable (Rigos et al. 2021). When the farmers notice changes in fish feeding, behaviour and mortality, they contact a competent veterinarian that starts the diagnostic procedure through fish necropsy examination, tissue sampling and bacterial isolation for the identification of the pathogen responsible for the symptoms and mortality observed in fish; subsequently, he requests the reference laboratory to perform antibiotic sensitivity tests to assess the sensitivity or resistance of the isolated bacterial strain to different drugs and define which one is more suitable to be used for the therapy of infected fish, appoints the preparation and purchase of medicated feed or antibiotics and oversees the fish feeding by farmers and farm staff.

The completion of the overall diagnostic procedure and the identification of the most appropriate antibiotic lasts too long, at least five to seven days, and the treatment usually starts too late when fish losses are already very significant (Zrnčić 2020). Moreover, the treatment of bacterial diseases in aquaculture is commonly metaphylactic, i.e. it is aimed to treat both the sick animals and the others in the group to prevent the spread of disease (FAO/WHO/OIE 2003). However, the indiscriminate use of antibacterial drugs can lead to the development of antimicrobial resistance in pathogen and non-pathogen bacterial strains, and the accumulation of chemical residues in fish tissues and the marine environment (Smith 2012).

To avoid all these unwanted bad consequences for the profitability of aquaculture, quality of the final products and health of consumers and the environment, one of the main procedures of the good aquaculture practices strongly recommended to control the spread of infectious diseases on the farm consists in the revaccination of fish, previously vaccinated in the hatchery when they are transferred and farmed in cages.

As occurs after the vaccination carried out in hatchery, also in this case only fish that are healthy at the time of revaccination in a cage will develop an optimal immunity

against the pathogen. **Therefore, the necessary preparation of fish for further vaccine administration should start two weeks before the planned revaccination:**

1. a thorough fish health examination should be carried out because the procedure of vaccination, potentially stressful for fish, may trigger the development of infections in fish with a precarious health state and consequently mortality during the prodromic period; if any disease is detected (bacterial or parasitic), fish should be adequately treated before be submitted to revaccination
2. fish could be fed a diet enriched with immunostimulants for a few days before the vaccination (it is not compulsory), to improve the fish immune response induced by the vaccine
3. fish should be fastened before vaccination and the time of fasting depends on the fish size and sea temperature the fasting may last 24 hours if fish are small and the sea temperature is high, whereas it should last more time if fish are big and the sea temperature is low; fastening is important because fish with empty digestive tract tolerate better handling and respond better to the anaesthetics
4. the optimal temperature for vaccination is 17-22°C: it would be advisable not to carry out the vaccination during the period of water temperature changes or when the sea temperature is higher than 24°C

4.4.1. Immersion revaccination

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A day before vaccination

1. all equipment needed for immersion vaccination should be cleaned and disinfected (tarpaulin, plastic bowls for immersion, balance, seines, bottles with oxygen, oximeter etc.)
2. a cage with a clean net to accept vaccinated fish should be prepared
3. a quantity (biomass) of fish to be vaccinated should be evaluated to prepare a sufficient quantity of vaccine
4. a vaccination plan with trained personnel should be set up so that each person should know what he will work and when

On the day of vaccination

1. remove the bottle of vaccine from the refrigerator and leave it to acquire the environmental temperature, shake it well to be sure that the content is evenly mixed, and dilute the content immediately after opening with farming water according to the instruction for use issued by the vaccine producer and mix it thoroughly (Fig. 35): the most commonly advised dilution is 1:10 (one litre of vaccine and 9 litres of farming water) but it may vary based on the vaccine producer and sometimes the dilution ration is 1:20; in general one litre of vaccine is sufficient for the vaccination of 100 kg of fish of above 5 grams

Figure 35. Vessel with the diluted vaccine for immersion revaccination

2. ensure that personnel involved in vaccination wear suitable protection such as rubber gloves and protective glasses
3. net of the cage with fish to be vaccinated should be shortened in half so fish that are going to be vaccinated become concentrated (Fig. 36)

Figure 36. Shortening of the nets in the cage meant for revaccination

4. a tarpaulin should be placed around the net and the oxygen supply provided; the oxygen saturation should be continuously monitored and the oxygen should be added to the tarpaulin in the case of oxygen concentration decrease (Fig. 37 and 38)

Figure 37. Placing of tarpaulin around the shortened net

Figure 38. Continous control of oxygen saturation in the tarpaulin

5. Fish in the tarpaulin should be slightly sedated with low doses of anaesthetics

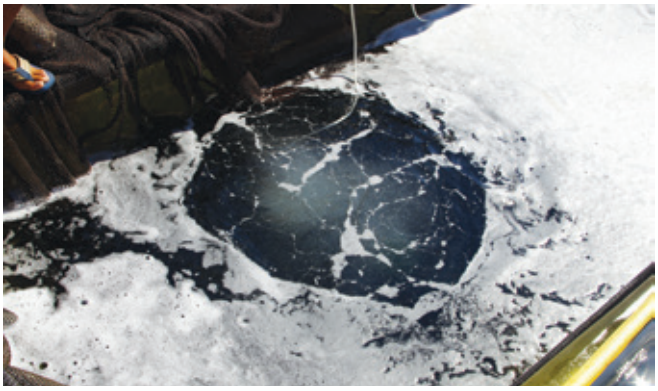

Figure 39. The addition of oxygen into tarpaulin with anaesthetic

6. batches of not more than 0.5 kg of fish should be netted out from the tarpaulin and holding water should be drained to prevent additional dilution of the vaccine (Fig. 41)
7. fish should be weighed to avoid the immersion of more fish than those permitted based on the prepared amount of vaccine

Figure 40. Immersion of fish in diluted vaccine

8. fish should be immersed into the diluted vaccine and kept there for 30 seconds (Fig. 40)

Figure 41. Weighing of vaccinated fish

9. after vaccination, the net with fish should be weighed (Fig. 41) and then directly released into a new cage (Fig. 42)

Figure 42. Releasing of vaccinated fish into a new cage

10. the waste vaccine should be discarded, according to the instructions of the producer

After vaccination

1. carefully observe the behaviour of vaccinated fish and slowly start to feed them again the next day

4.4.2. Injection revaccination

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The preparation of fish to be submitted to revaccination through injection is similar to that which is carried out before fish revaccination by immersion. **Only a few additional precautions should be taken into account:**

1. before vaccination, fish should be graded based on their size to increase the speed and accuracy of the vaccination procedure
2. fish smaller than 15 g are not appropriate for injection vaccination
3. fish should be fastened 24 hours before vaccination when the sea temperatures are higher than 19 °C or 3 days if sea temperatures are lower than 18 °C; this aspect is very important because the presence of feed in the stomach or intestine may lead to the vaccine injection into the fish digestive system
4. the vaccine should be stored at 4-8 °C

A day before vaccination

1. all equipment needed for the injection vaccination (Fig. 43) that could come into contact with fish should be cleaned and disinfected (tarpaulin, seines, vaccination tables, pipes, pumps, automated syringes, sufficient number of needles, vessels for anaesthesia etc.)

Figure 43. Prepared and cleaned equipment needed for revaccination with injection on the farm

2. if the vaccination machine is to be used, it should be prepared and operated by a trained techniciani
3. a cage with a clean net to accept vaccinated fish should be prepared
4. the quantity (biomass) of fish to be vaccinated should be determined to prepare a sufficient quantity of vaccine
5. a sufficient quantity of approved anaesthetic should be prepared
6. a vaccination plan with trained personnel should be set up so that each person should know what he will work and when
7. the injection device must be calibrated (Fig. 44)

Figure 44. Calibrated injection device

8. an appropriate size of needles should be determined according to the size of the fish, the thickness of the abdominal wall and the injection point: usually needles long of 3 mm are used for fish of 25-40 g, 4 mm for fish of 40-80 g, 5 mm for those weighing more than 80 g and the diameter of needles is 0.6 mm
9. personal should be familiar with the safety procedures and instructions in case of self-injection; if a team is not equipped with adrenalin auto-injectors, a physician should be readily available

On the day of vaccination

1. check the vaccine before use: it should appear homogeneous after shaking the bottle (Fig. 45)

2. the vaccine should be taken out from the refrigerator and maintained at a temperature ranging from 15 to 20°C so that it is easier to work with it; it should be protected from high temperature and direct light

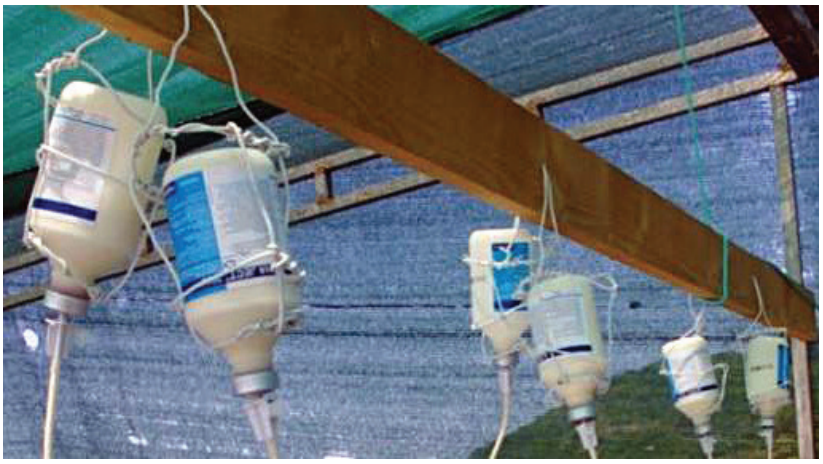

Figure 45. The homogenous appearance of vaccine

3. fish should be gathered by shortening nets and putting tarpaulin with continuous oxygen supply and sedated with a low dosage of anaesthetics (Fig. 46)

Figure 46. Shortening of the net and placing tarpaulin

Figure 47. Continuous oxygen supply into tarpaulin and netting of small batches for vaccination

4. a vessel with anaesthetic should be prepared, and then small batches of fish should be netted out of the tarpaulin (Fig. 47) and immersed in anaesthetic for 40 to 120 seconds (Fig. 48)
5. the anaesthetic solution should be changed according to manufacturer instructions
6. a suitable number of fish should be anaesthetized to enable all of them to be vaccinated within 3 minutes and avoid high losses due to the stress caused by manipulation

Figure 48. Immersion in anaesthetic

7. fish to be vaccinated should be still on the table (Fig. 49)

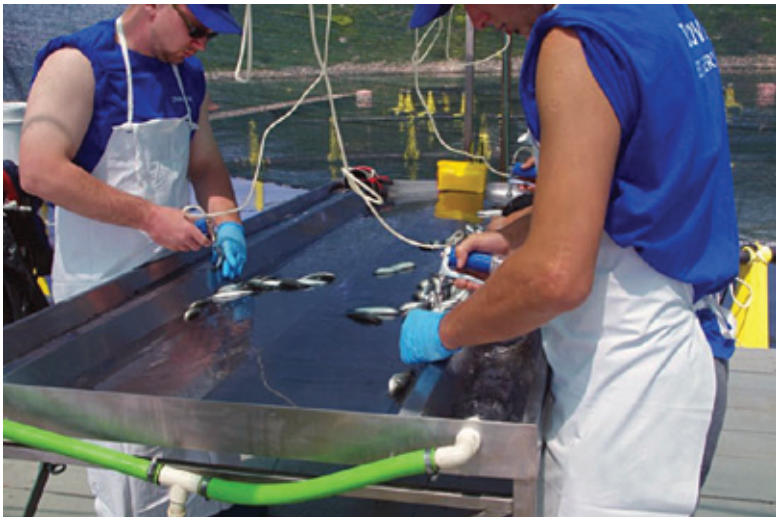

Figure 49. Injection of the vaccine on the table for vaccination

8. work speed should be adjusted based on the skills of operators and the injection should be placed at an angle of 90 degrees to the abdominal wall and gently pressured to avoid injuries in the point (Fig. 50)
9. the vaccine should smoothly enter the abdominal cavity and the syringe should not be taken out before the whole dosage of the vaccine has been injected
10. for example, the appropriate point for injection of sea bass is in the midline about one and a half pelvic fin lengths posterior to the base of the pelvic fin
11. the vaccine should be homogenous all the time during the vaccination procedure

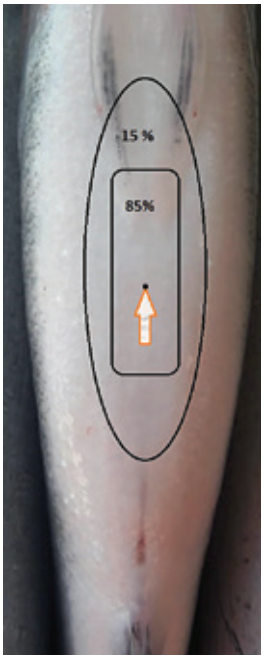

Figure 50. Determination of the point for injection of vaccine

12. an opened bottle should be used within 12 hours
13. the needle should be changed when it becomes damaged or blunt, or after 2000 fish have been vaccinated
14. fish scales should be regularly removed from the needles to avoid changes in needle length

Figure 51. Manipulation with fish during vaccination

15. during the vaccination, fish should be gently manipulated to avoid stress or damage (Fig. 51)
16. operators should use clean gloves and change them regularly to avoid contamination

17. regular control of the efficacy of vaccine administration should be controlled by gentle pressure on the fish's abdominal wall at the point of injection to detect the presence of whitish liquid, furthermore, some specimens should be sacrificed for checking the presence and quantity of vaccine in the body cavity to be able to correct the procedure
18. inappropriate vaccine administration may cause injuries of intestine or other internal organs in fish
19. after vaccine administration, fish should be released to the recovery tank or directly to a new cage (Fig. 52) and they should start to swim very quickly

Figure 52. Release of vaccinated fish to the new cage

After vaccination

1. fish should fully recover in a week
2. small amounts of feed should be offered to vaccinated fish 1-day post-vaccination during the summer or 2-3 days post-vaccination during the winter: the feed does not pass through the intestine during the first days post-vaccination, therefore it may ferment causing enteritis in fed fish
3. post-vaccination mortality is usually low and most fish start to eat normally within a week

References

FAO/OIE/WHO. 2003. Joint FAO/OIE/WHO Expert Workshop on Non-Human Antimicrobial Usage and Antimicrobial Resistance: *Scientific assessment*. Geneva, December, 1–5 2003.

Pharmaq Zoetis. Vaccination Manual. https://www.pharmaq.com/en/pharmaq/our-products/

Rigos, G., Kogiannou, D., Padrós, F., Cristofol, C., Florio, D., Fioravanti, M., & Zarza, C. 2021. Best therapeutic practices for the use of antibacterial agents in finfish aquaculture: A particular view on European seabass (*Dicentrarchus labrax*) and gilthead seabream (*Sparus aurata*) in Mediterranean aquaculture. *Reviews in Aquaculture*, 13, 1285-1323.

Smith, P. 2012. Antibiotics in aquaculture: reducing their use and maintaining their efficacy. In *Infectious Disease in Aquaculture* (pp. 161-189). Woodhead Publishing.

Zrnčić, S. 2020. Correct diagnostics: prerequisite for prudent and responsible antimicrobial administration. *Asian Fisheries Science*, 33, 27-32.

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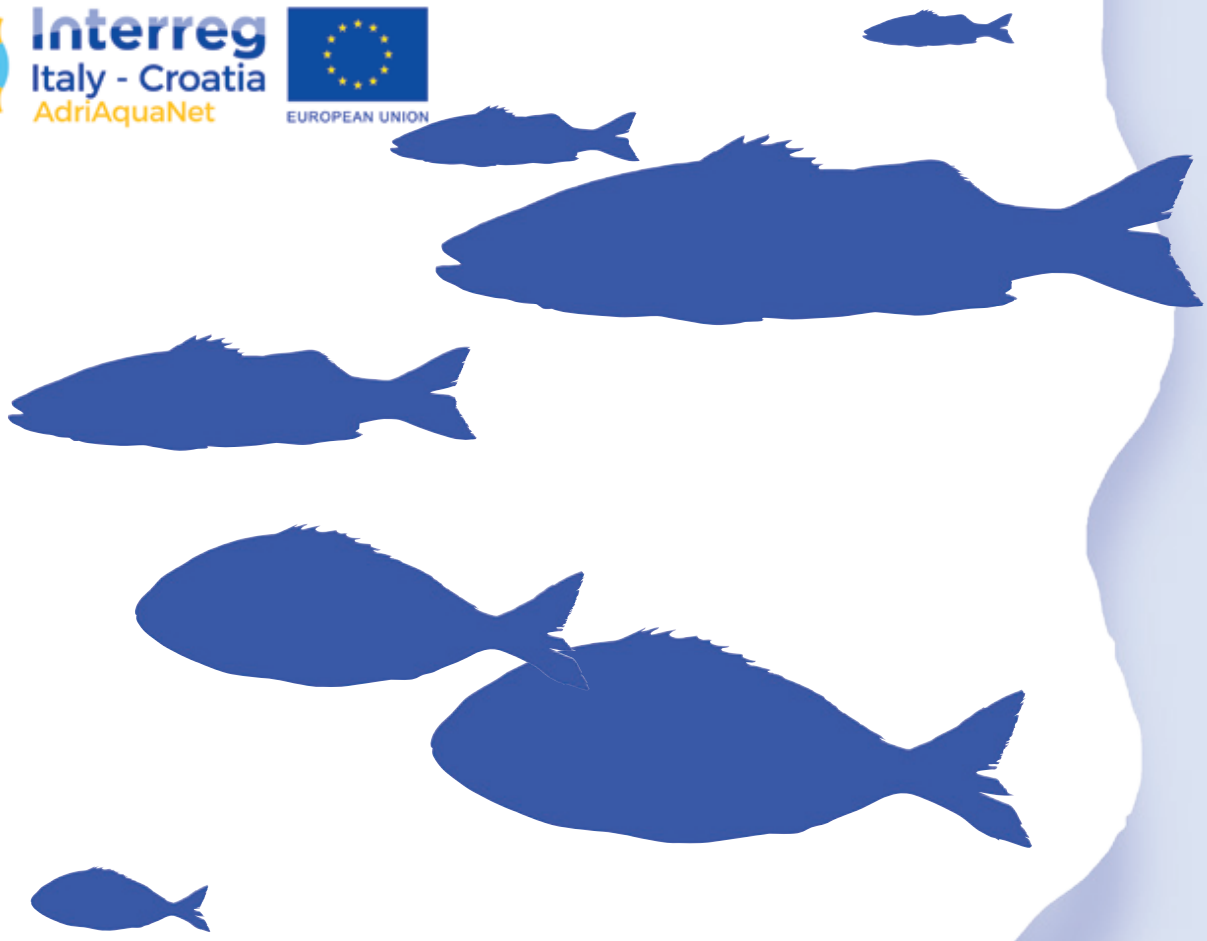

Adriatic mariculture supplies high-quality fish products to the local markets as well as the markets of the neighbouring countries. Aiming to ensure the further economic development of this sector based on environmental and social sustainability, scientists and producers on both sides of the Adriatic Sea; Italy and Croatia launched the project **"STRENGTHENING INNOVATION AND SUSTAINABILITY IN ADRIATIC AQUACULTURE" - ADRIAQUANET**.

ADRIAQUANET CONSORTIUM is composed of scientists from seven research institutions, four production organizations and breeders' associations from Italy and Croatia. The activities were financed from the Interreg Italy-Croatia 2014-2020 program, until June 2022. The coordinator of the consortium is prof. Marco Galeotti from the the University of Udine, Italy.

THEY DEFINED **THREE MAIN GOALS TOGETHER:**

FISH FARMING: improvement of fish farming by introducing innovations in feeding technology and disposal of waste materials.

FISH HEALTH: strengthening resistance to diseases by applying new autogenous vaccines, probiotics and natural medicinal substances. The application of the principle of fish welfare is a strategic determinant in the prevention of the occurrence of diseases.

MARKETING: assessment of the quality of farmed fish with welfare principles in ecologically favourable conditions based on the analysis of hygienic, sensory and nutritional parameters and its promotion as the development and promotion of new fish products that will meet the needs of the market