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Vaccines and immune protection of principal Mediterranean marine fish species

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ABSTRACT

This review describes and summarizes the knowledge on established and experimental vaccines developed against viral and bacterial pathologies affecting the most important farmed marine finfish species present in the Mediterranean area, namely European seabass *Dicentrarchus labrax*, sea bream *Sparus aurata*, turbot *Psetta maxima* and meagre *Argyrosomus regius*. The diseases that have been recorded in seabass, sea bream and meagre are caused by bacteria *Vibrio anguillarum*, *Photobacterium damselae*, *Tenacibaculum maritimum* as well as by viruses such as Viral Encephalopathy and Retinopathy/Viral Nervous Necrosis and Lymphocystic disease. The main pathologies of turbot are instead bacteriosis provoked by *Tenacibaculum maritimum*, *Aeromonas* sp. and *Vibrio anguillarum*, and virosis by viral hemorrhagic septicaemia virus. Some vaccines have been optimized and are now regularly available for the majority of the above-mentioned pathogens. A measurable immune protection has been conferred principally against *Vibrio anguillarum, Photobacterium damselae* sub. *piscicida* and VER/VNN.

1. Introduction

The latest State of World Fisheries and Aquaculture edited by the Food and Agriculture Organization reported that the global fish production accounted for 171 million tonnes in 2016. The aquaculture sector alone has represented the 47% of the total production and, after a steadily growing trend, is for the first time set to contribute half of the fish food consumed worldwide, consisting in approximately 80 M tonnes and a first-sale value estimated at USD 231.6 billion [1]. This envisages the importance of producing high quality seafood while respecting fish welfare and the environment.

The Mediterranean basin is an area where fish farming has been practised for over two millenia. In the last 40 years some teleost species have been introduced in aquaculture and produced in large amounts, namely the seabass (*Dicentrarchus labrax*, 192.000 t), sea bream (*Sparus aurata*, 186.000 t), turbot (*Psetta maxima*, 59.000 t), and recently the meagre (*Argyrosomus regius*, 23.000 t) (data from http://www.fao.org/fishery/en as of September 2019). As for any other vertebrate species, an efficient vaccination is a key component to combine the necessary requirements for a sustainable and healthy farmed fish production. Many efforts have been and are being made in investigating fish immunobiology to gather the knowledge necessary for an efficient vaccination. In fish, this is a particularly difficult task due to the many species-specifics biotic and abiotic parameters requiring optimization for each particular pathogen. Target pathogens are researched for the best antigen component(s); vaccination strategies (i.e. the role of adjuvants on immunogenization efficiency) [2,3] and most effective delivery methods [4] must also be enhanced, or low antibody titers and a time-limited immune protection may otherwise result.

Fish pathogens are of various origin (i.e. viruses, bacteria, parasites), or can become pathogenic when immune protection is decreased due to stressful conditions impacting the animal's physiology [5,6]. The first studies on antigen-driven antibody responses in teleost fish date back to 1942 [7], and knowledge in immunobiology has increased dramatically along with farmed fish production since then. This has led to tools that allow for a better control of vaccination strategies and the associated immune responses: as a result, vaccines against bacterial pathogens and, recently, viral pathologies are now available for the above listed aquaculture-relevant teleost species. The list of authorized vaccines cited in the present review was obtained by querying the databases of the Italian Ministry of Health dedicated to veterinary medicinal products (https://www.vetinfo.it/j6_prontuario/public/) and the Heads of Medicines Agencies (https://www.hma.eu/fileadmin/dateien/Veterinary_medicines/CMDv_Website/Procedural_guidance/Miscellaneous/Veterinary_Medicinal_Products_intended_for_fish_2019.xlsx -last updated on June 21, 2019) for fish species and analyzed for the induced immune activities, when available.

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2. Viral pathologies

2.1. Nodavirus

The most devastating teleost-specific viral pathogen is represented by a retrovirus of the genus *Betanodavirus* (also known as Nervous Necrosis Virus - NNV), family *Nodaviridae* [8,9]. NNV is an RNA virus whose genome is composed of two single-stranded RNAs encoding for an RNA-dependent RNA polymerase and a capsid protein possibly involved in inducing cell death, respectively [10]. A third subgenomic RNA encoding for additional structural protein is synthesized during viral replication [11].

The first outbreak was recorded in 1985, while the virus itself was purified from striped jack *Pseudocaranx dentex* larvae in 1992 [12,13]. It has been isolated from over 70 freshwater and marine teleost species wordlwide so far, where it was demonstrated to cause viral encephalopathy and retinopathy/viral nervous necrosis (VER/VNN) [8]. Due to climate change effects on fish physiology and pathogen viability, such a count is likely to rise in the near future, especially because NNVs thrive in warm conditions [14].

The clinical signs of VER vary depending on fish species. Early signs of infection can be generally identified in atypical skin pigmentation (i.e. darkening or whitening) followed by abnormalities in the feeding and locomotive behaviours (i.e. anorexia, lethargy on the bottom, erratic spiralling/whirling swimming pattern) reflecting the impairment of the nervous system as well as hyperinflation of the swim bladder and intracerebral hemorrhages [4,8,15–17]. On a microscopical level, vacuolated and necrotic cells are found in the spinal cord, brain and retina of larval and juvenile stages [8] and 20–34 nm icosahedral, naked viruses can be observed by electronic microscopy either free or membrane-bound [13].

Four viral genotypes, namely barfin flounder nervous necrosis virus (BFNNV), tiger puffer nervous necrosis virus (TPNNV), striped jack nervous necrosis virus (SJNNV) and red-spotted grouper nervous necrosis virus (RGNNV) have been described and *in vitro* shown to differ in terms of optimal growth temperature (i.e. 15–20 °C, 20 °C, 20–25 °C and 25–30 °C, respectively) [18]; a host-free nomenclature scheme was also suggested based on phylogenetic analyses of the coat protein gene [19]. RGNNV is particularly pathogenic to farmed European seabass and a variety of warm-water fish species (e.g. groupers) [8]; SJNNV was initially assumed to be limited to Japanese waters but was recently found in southern European species such as *Solea senegalensis*, *Sparus aurata* and *Dicentrarchus labrax* [20].

NNVs entered the European aquaculture farms in the 90's by infecting *D. labrax* larvae [21]. Infections caused by RGNNV and SJNNV genotypes and, recently, reassortant RGNNV/SJNNV and SJNNV/RGNNV viruses rapidly spread to several perciformes and pleuronectiformes species (e.g. European seabass, sea bream, meagre, Senegalese sole, turbot) resulting in elevated mortality rates of larval, juvenile and caged grown-out stages and consequently in large economic losses in the whole basin [22–26]. Much literature is available on *D. labrax*, the pathology and its serotypes [4,27,28] as well as on immune responses against the infection [29–32].

NNVs can spread both vertically and horizontally. Vertical transmission occurs through the gonadal tissue via infected eggs and sperm, where the virus replicates in response to stressful conditions [12,33]. Horizontal transmission is arguably the hardest to control and/or limit since it occurs during outbreaks, whenever healthy fish come in contact with infected individuals, tissue homogenates, asymptomatic carriers (e.g. apparently healthy invertebrates such as bivalves, crustaceans, brine shrimps and rotifers) or even infected rearing equipment [34–37]. Such a transmission route was first demonstrated in seabass by cohabitation experiment [4]. NNVs infect immuno-incompetent larvae and juveniles by entering the nasal cavity and spreading through the olfactory nerve and bulb, brain, medulla, spinal cord and retina [12].

Traditional methods of detection involve the isolation of the virus from permissive cell cultures (e.g. SSN1) with subsequent immunological assays, which were initially complicated by poor knowledge of antibody response kinetics [4,8]. Molecular biology techniques have increasingly been employed since early 2000s [38] and allowed detection in non-neuronal tissue, including gills, kidney, liver, digestive tract, heart, spleen, intestine, gonads and fins. These consists in *in situ* hybridization (ISH) and amplification-based methods (RT-PCR, qPCR, LAMP) [39,40]. They offer several advantages in terms of costs, rapidity and sensitivity, especially when highly conserved regions of the coat protein encoding gene are targeted [41]. Previous papers have reported the use of primer pairs that allow the discrimination of Mediterranean and Atlantic NNV strains [42]. It is not advisable that primers be designed on the RNA2 T4 variable region [43]: as an example, Johansen and co-workers failed to obtain any result when trying to amplify a turbot genome with primers designed on an Atlantic halibut nodavirus template [44].

2.2. Vaccines and immune protection against nodavirus

The severity of the pathology induced by NNV in seabass *D. labrax*, which in certain conditions can exterminate fish stocks, led to much effort being put into finding strategies for an efficient vaccination.

Available literature suggests that VER/VNN virus can be immunogenic in seabass, and thus the possibility of preparing an efficient vaccine is real. However, additional factors might affect vaccine efficiency such as the number of diverse virus genotypes/serotypes [45], the environmental variables in the diverse Mediterranean sub-areas [28] and the many susceptible fish species that contribute to disease spreading.

Early attempts tested four 15 AA-long peptides of VER/VNN capsid protein for potential immunogenicity [46]. The N-terminal peptide appeared to be the most potent in eliciting the production of NNV antibodies when intramuscularly (IM) injected. Despite discretely positive results were obtained, the production of a vaccine based on synthetic peptides for aquaculture applications may not be upscalable because of costs/benefits reasons.

Virus-Like Particles (VLPs), namely uninfectious particles made of a single type of coat protein and random cellular RNA that are antigenically identical to the native virus, were produced in baculovirus and tested as IM-delivered immunogens at 1, 0.2, 0.1, 0.05, 0.01, 0.005 and 0.001 μg/μl concentrations. Results were promising: both immune response and protection against homologous wild-type viral challenges were dose-dependent. The experimental group that received the highest dose had a mean mortality of only 8.5% and the lowest PCR-detectable quantity of viral RNA [47].

Recent works described attempts of vaccination and immune response of seabass against VER/VNN with a chitosan-encapsulated DNA for mucosal delivery. The vaccine either failed to induce NNV-specific antibodies or up-regulate IgM gene expression in the posterior gut but succeeded in up-regulating the expression of genes involved in cell-mediated cytotoxicity and the inflammatory interferon pathway [48].

Others clearly showed that whole inactivated virus induced specific IgM titers and some degree of protection upon intraperitoneal (IP) administration [29]. An efficient uptake of the virus through the gills epithelium after immersion immunization was also demonstrated, but neither a systemic immune response nor an *in vivo* protection with respect to IP vaccination was induced [49]. Since injection-delivered NNV vaccines are only feasible on grown-out specimens and the disease is normally associated with outbreaks in larvae and juvenile stages, antigen(s) administration by immersion would by far be the preferred delivery route in stress-prone fish species. However, as of now, it is not satisfactorily efficient and further work is required [50]. A promising alternative, so far tested on grouper *Epinephelus coioides*, consists in the bioencapsulation of NNV capsid protein into *Artemia salina* [51]. The most efficient approach, though, still consists in vaccinating the broodstock rather than larvae/juvenile fish: previous successes were

obtained in potato grouper *Epinephelus tukula*, where the virus was undetectable in the eggs of IM-injected fish 5 months post vaccination [52].

As anticipated above, the sea bream *Sparus aurata* is not significantly affected by the pathology, although recently RGNNV/SJNNV and SJNNV/RGNNV genotype reassortants were shown to induce mortality [26]. Sea bream thus represents a reservoir for the VER/VNN, since the virus is present and recognized by its immune system [33,53].

Turbot species *Psetta maxima* (formerly known as *Scophthalmus maximus)* belonging to the Scophthalmidae family is affected by VER/VNN too but only few studies focused on it. In these, the pathology was characterized in terms of histopathology, immunohistochemistry (IHC), cell cultures and RT-PCR amplification and sequence analyses of the RNA2 segment. For completeness of information, a positive staining with SJNNV-specific antibody was found in both nodavirus and pre-immune serums [44].

The transcripts affected by a challenge with 10^6 $TCID_{50}$ ml^{-1} (tissue culture infective doses) nodavirus were analyzed by at-the-time innovative bioinformatic methods using genomic libraries constructed from liver, kidney and gill sampled from larval specimens. The most modulated expressed sequence tags (ESTs) belonged to the category of cell/organism defense and apoptosis-related processes; as for the immune response category, ESTs homologous to immunoglobulins and their receptors, T-cell receptors, MHC class I and II and related genes, chemokines and interferon-related genes were annotated [54].

The responses to nodavirus infection in terms of innate immune activities as well as the production of nitric oxide *in vivo* and *in vitro* were also studied by means of qPCR and the Griess reaction, as described in Ref. [55]. Both the expression of cytokines (TNF-α, IRF-1 and Mx) and production of nitric oxide significantly increased by 3–7 days post infection and in more than 60% of infected fish, respectively. This highlighted the indispensable contribution of inflammation as a defense mechanism in organism infected by nodavirus [56].

At last, by performing a cohabitative experimental challenge, horizontal transmission between turbot and Atlantic cod *Gadus morhua* was demonstrated over a 4-month period. From an ecological perspective this was especially significant, as wild cods in proximity of turbot hatcheries and farms may be at risk, despite the study did not directly demonstrate NNV's ability to replicate in cod [57].

As for VER/VNN infecting the meagre *Argyrosomus regius,* poor knowledge is available, also in terms of the species' immune defences [58,59]. However, the presence of both SJNNV and RGNNV genotypes has been detected [60].

Despite several efforts had been made, until 2018 no vaccination against VER/NNV was commercially available. Only very recently was a vaccine against RGNNV formulated to specifically protect seabass and, possibly, the other described species (Table 1). The IP-injected vaccine consists in > 0.07 antigenicity units/dose of inactivated RGNNV genotype emulsified in liquid paraffin as adjuvant. Efficacy has been documented by challenges up to 12 months post vaccination, however, the effects on the immune system of seabass has not been reported so far.

2.3. Other viruses

With the exclusion of sea bream that can be sensitive to a lymphocystis-inducing DNA virus belonging to the Iridovirus genus [61], no other viral species have been listed as pathogens for the considered Mediterranean species, and no vaccines are consequently available.

3. Bacterial pathologies

3.1. Vibriosis

Vibriosis is a pathology principally carried by the Gram-negative halophilic *Listonella anguillarum* (formerly *Vibrio anguillarum*), and less frequently by other related species *Vibrio alginolyticus*, *Vibrio harveyi*, *Vibrio ordalii, Vibrio vulnificus*. The genus *Vibrio* alone encloses more than 140 species (data from http://www.bacterio.net/vibrio.html as of July 2019). All the fish species cultured within the Mediterranean basin are affected by vibriosis, which is the most important disease after VER/VNN in Mediterraneam aquaculture [62], with seabass being the most sensible. Because mollusks and crustacean can suffer from high mortalities caused by *Vibrio* spp. outbreaks and bacteria can survive in aquatic environment in a host-free manner, integrated multi-trophic aquaculture systems can be severely hampered by vibriosis [63]. Bacteria of the genus *Vibrio* are usually present in environmental microflora and are generally tolerated, but may become infective when the fish immune defences are impaired by stress conditions [64]. Due to the microorganisms' life strategies and the predilection for high water temperatures, vibriosis never occurs in freshwater aquaculture -unless fish are fed feeds of marine origin- and therefore is not recognized as a problem in sub-Arctic regions [63].

The virulence of *Vibrio* spp., which is manifested through a generalized septicaemia, haemorrhagic intestines, body cavity, spleen and muscle, exophthalmia and eroded skin and fins [65], is caused by several factors. Since the primary transmission mode of Vibrionaceae is through the water and occasionally via oral ingestion, virulence factors can be identified in the products of genes related to adherence, entrance, multiplication and avoidance of host defences [66]. The cell surface structure, with its flagella, adhesive factors to fish mucus, capsular polysaccharides and lipopolysaccharides certainly play a major role in triggering the infection, as motility, colonization and increased resistance to phagocytosis are acquired [67]. The secretion of toxic extracellular products (ECPs) and the ability to sequester iron from host transferrins are also involved [63].

3.2. Vaccines and immune protection against genus Vibrio

Fish vibriosis is an apportunistic pathology, since it is generally tolerated in healthy conditions, but may became very dangerous and induce mass mortalities when stressful conditions make fish prone to infection [64]. In addition, the bacterium can evade immune responses by inhibiting key physiological pathways [68]. The earliest attempts to produce effective vaccines against fish vibriosis date back to 1991 [69]. The first vaccine formulations for *L. anguillarum* has been reported in 1998 [70], and subsequently vaccination against vibriosis became a successful practice in Mediterranean aquaculture [71]. At present, a list of registered vaccines is available for serotypes 01, 02α, and 02β (Table 1). They can be administered by immersion and/or intraperitoneal injection, and with a formulation optimized for oral vaccination. Vaccines for vibriosis are generally constituted by inactivated whole-cell bacterins. LPS -the main antigenic component- is either emulsified with mineral oil and optimized for intraperitoneal administration or adjuvanted with TNFα [72,73], IL-1β [74] and aluminium [75] for mucosal/intraperitoneal vaccination. The *Vibrio* vaccine antigens can be also included in pelleted food, and a study elucidated RNA profiling after oral vaccination of seabass [76]. The *Vibrio* antigenic formulations, given as an IP commercial vaccine, after few weeks are able to induce specific IgM serum titers, *in vitro* IgM production by memory B cells and *in vitro* antigen-induced leukocyte proliferation [77]. Some vaccine formulations have been designed to be monovalent and specific for vibriosis, others are bivalent for vibriosis/pasteurellosis, and the duration of immunity after vaccination, when tested, may vary from 6 weeks to one year.

A general problem in fish vaccination is the delivering site of the antigenic mixture. IP administration is successful in inducing immune responses and protection but can be only performed on fish of an appropriate body size. In addition, IP administration may be stressful, making fish prone to subsequent infections, and some side effects of intraperitoneal vaccination against vibriosis have been investigated [78].

Table 1
Table reporting commercial vaccine names, target specificity with regards to Mediterranean species only and immunization details.

Vaccine name	Target species	Antigen (serotype/strain when reported)	Duration of immunity (when reported)	Delivery route	URL
ALPHA DIP 2000	*D. labrax*	• Inactivated L. *anguillarum* (serotype 01) • Inactivated *Phdp*	N/E	Immersion/IP	https://aquavet.gr/products/vaccines/alpha-dip-2000/?lang=en
ALPHA DIP VIB	*D. labrax*	• Inactivated L. *anguillarum* (serotype O1, strain AL 112)	N/E	Immersion/IP	https://mri.cts-mrp.eu/human/downloads/ES_V_0251_001_PAR.pdf
ALPHA JECT 2000	*D. labrax*	• Inactivated L. *anguillarum* (01) • Phdp	N/E	Injection	https://aquavet.gr/products/vaccines/alpha-ject-2000/?lang=en
ALPHA JECT MICRO 1 NODA	*D. labrax*	• Inactivated RGNNV strain ALV1107	1 year	Injection	https://www.pharmaq.no/sfiles/2/54/9/file/product-info_alpha-jet_micro-1-noda_english_2018-9.pdf
AQUAVAC PHOTOBAC PRIME	*D. labrax* *S. aurata*	• Inactivated *P. damselae* (strain Pr85)	N/E	Injection	https://thefishsite.com/focus/msd-animal-health/merck-product-overview-vaccines/aquavac-photobac-prime
AQUAVAC VIBRIO VAB[a]	*D. labrax*	• Inactivated L. *anguillarum* (strain 78 SKID) • Inactivated *Vibrio ordalii* (strain MSC 275)	N/E	Immersion/IP	https://thefishsite.com/focus/msd-animal-health/merck-product-overview-vaccines/aquavac-vibrio
AQUAVAC VIBRIO ORAL[a]	*D. labrax*	• Inactivated L. *anguillarum* (strain 78 SKID) • Inactivated *Vibrio ordalii* (strain MSC 275)	N/E	Oral	https://thefishsite.com/focus/msd-animal-health/merck-product-overview-vaccines/aquavac-vibrio-oral
AQUAVAC VIBRIO PASTEURELLA	*D. labrax*	• Inactivated L. *anguillarum* (biotype I and II) • Phdp	N/E	Injection	https://thefishsite.com/focus/msd-animal-health/merck-product-overview-vaccines/aquavac-vibrio-vibrio-pasteurella
ICTHIOVAC STR	*P. maxima*	• Inactivated *Streptococcus parauberis* (strains RA-99.1 and AZ-12.1)	2 years	Injection	https://mri.cts-mrp.eu/human/downloads/ES_V_0109_001_FinalPI.pdf
ICTHIOVAC VR/PD	*D. labrax*	• Inactivated *P.damselae* (strain DI 21) • Inactivated L. *anguillarum* (serotypes O1, O2α and O2β)	N/E	Injection	https://www.hipra.com/portal/it/hipra/animalhealth/products/detail/icthiovac-vr-pd
ICTHIOVAC-PD PASTEURELOSIS DORADA	*S. aurata*	• Inactivated *P. damselae* (strains DI 21 and LT-1)	5 months	Immersion/IP	https://cimavet.aemps.es/cimavet/pdfs/es/ft/1466+ESP/FT_1466+ESP.pdf
ICTHIOVAC TM	*P. maxima*	• Tenacibaculum maritimum LPV 1.7 (serotype 02)	6 months	Injection	https://www.hipra.com/portal/en/hipra/animalhealth/products/detail/icthiovac-tm
VIBRI-FISHVAX[a]	*D. labrax* *S. aurata*	• Inactivated L. *anguillarum* • Vibrio ordalii	10–12 months	Immersion/IP	http://www.medpills.it/farmaci/schede/vibri-fishvax-1fl-1000ml.html

N/E: Not established.
[a] The vaccine was also found to confer immunity against vibriosis in rainbow trout *Oncorhynkus mykiss*.

3.3. Pasteurellosis

Pasteurellosis/photobacteriosis is a septicemic disease caused by the facultative intracellular, halophilic, Gram-negative bacteria *Photobacterium damselae* subsp. *piscicida* (Phdp) (formerly *Pasteurella piscicida*) [79]. The disease is also known as pseudo-tuberculosis because of the tubercle-like processes appearing in both wild and cultured fish that suffer from chronic infections.

Pathogenicity in this bacterial species is conferred by several traits, namely i) a lipopolysaccharide capsular layer, the formation of which is affected by temperature conditions and nurients availability, that confers *in vivo* adhesion to the intestine as demonstrated in sea bream *Sparus aurata*, seabass *Dicentrarchus labrax* and turbot *Psetta maxima* [80]; ii) the replication-free *in vivo* survival in non-specialized phagocytic and epithelial cells for at least 7 days in several fish species (i.e. sea bream, carp *Cyprinus carpio*, catfish *Ictalurus* sp., fathead minnow *Pimephales promelas* and chinook salmon *Oncorhynchus tshawytscha*) [81–83]; iii) siderophores-mediated iron sequestering capabilities using hemin and hemoglobin as sources [84]; iv) the secretion of ECPs with haemolytic and phospholipase activities specifically toxic to teleosts, as shown for seabream, rainbow trout *Oncorhynchus mykiss* and turbot [85]; v) the secretion of a plasmid-encoded exotoxin (AIP56) activating the host cell-death pathway that leads to apoptosis and necrosis of macrophages and neutrophils [86,87]; vi) consumption of the complement providing resistance to serum, as seen in sea bream, seabass and red porgy *Pagrus pagrus* [88].

The disease was described in Japan in yellowtail *Seriola quinqueradiata* [89] and later found to restrict the expansion of intensively-cultured Mediterranean species such as seabass, sea bream and, to a lesser extent, turbot [2,90]. Pasteurellosis has broad host range distribution, causes high mortality rates and economic losses and is one of the most threatening diseases in Mediterranean aquaculture [91]. Elevated densities, water temperatures higher than 23 °C, salinities ranging between 20 an 30‰, low oxygen concentrations and poor water quality all contribute to the spreading of the pathology [92,93].

Despite some papers indicate that aquatic free-living Phdp remains in a culturable form for only 4–5 days [94,95], others have demonstrated that “dormant” cells maintain their pathogenicity and can resume whenever nutrients become available [96]: the water is therefore a primary reservoir. Virulent strain antigens are transmitted through the skin and the branchial epithelium: here, secreting cells are observed following vaccination, while the systemic compartment (head kidney and spleen) is only slightly affected [97,98].

Because neither gross external signs (i.e. darkening of body color) nor surface lesions (i.e. haemorrhagic areas in the head and gills) generally occur [99], a rapid diagnosis is of paramount importance. Standard microbiological procedures can be hampered by the slow growth of this species; serological techniques based on specific antibodies (e.g. BIONOR Mono-kits) as well as amplification-based methods (e.g. Diatheva PCR Detection Kit) were developed and instead allow for detection in as short as 1 and 2.5 h, respectively. The latter claims to allow the discrimantion of the subsp. *damselae* from the subsp. *piscicida* thanks to the amplification of a 297-bp long specific target region. Phdp can be also detected by measuring levels of serum nitrate and citrulline (or any other reactive nitrogen intermediate) as proxies of nitric oxide responses, which are known to occurs in mammals and fish as antimicrobial mechanisms [100]. This approach was confirmed on sea bream with the nitrate reductase method [3].

The ever increasing bacterial resistance caused by antibiotic treatments against several pathologies -as reported for more than 20 years by a multitude of authors [101]- emphasise the role of vaccination in the prevention of pasteurellosis [97].

3.4. Vaccines and immune protection against Photobacterium damselae subsp. Piscicida

Potential vaccine candidates against Phdp have been thoroughly tested for efficacy, preferred route of administration and novel identification methodologies.

The protective efficiency of different vaccine formulations, all based on formalin-inactivated whole Phdp cell strain 249/I99 was compared after an homologous IP-delivered challenge in 2 g body weight (BW) seabass. The vaccine was delivered orally (microincapsulated in calcium alginate microspheres coated by chitosan), by immersion -either monovalent or bivalent, in solution with an equal dose of *L. anguillarum*- and by IP injection, and evaluated for 14 days after a 40-day immunogenic period. IP injection resulted in the most effective route, with a cumulative mortality of only 16% compared to 91% of the controls. Relative percent survival (RPS) values of the other methods were equivalent even though the feed-administered oral formulation gave promising results to build on, especially because of the lower levels of stress associated to delivery [102].

An alike administration route was used in a multi-factorial study aimed at evaluating humoral immune responses of larvae spawned from vaccinated broodstock as well as optimal vaccination age in sea bream. Some degree of immune protection (later detailed as anti-protease activity, lysozyme activity and total immunoglobulin levels) [103] was maternally transferred to embryos and susceptibility tests consisting in virulent baths of 10^6, 10^4 and 10^2 Phdp cells ml^{-1} were better dealt with by larvae from immunised broodstock. In addition, 89-day old larvae immunised with either formalin-inactivated treated Phdp (10^6 cells ml^{-1}) or *E. coli* LPS (50 mg ml^{-1}) displayed higher anti-protease activity, lysozyme levels, total immunoglobulin levels and Phdp-specific antibody titres compared to respective control groups, overall resulting in lower mortality. The authors demonstrated that baths can be successfully used to immunize sea bream as early as 90 days post hatch, an age at which larvae are already immuno-competent, preferably in combination with broodstock vaccination.

With regards to IgT and its role in mucosal immunity, its modulation was analyzed at the gene expression and protein levels in sea bream and then compared to IgM data [104]. Following an oral vaccination with Phdp and subsequent challenge with the homologous pathogen, IgT antibody levels significantly increased in mucus but remained unchanged in the serum, showing an exactly opposite trend to that of IgM. The soluble form of the IgT transcript were also statistically up-regulated in intestine, spleen and head kidney tissues. Interestingly, expression kinetics of the two immunoglobulins were shown to differ depending on multiple factors such as type of pathogen, immunization, challenge, tissue and time after challenge.

Non-specific immune response was also studied [105]. Three differently-inactivated bacterins were prepared from Phdp strain 94/99 and used to immunize 5 g BW sea breams via short baths (60-s long). The expression of some pro-inflammatory cytokines (namely IL-1β, IL-Ir2, TNF-α and COX-2) was upregulated as early as 1 day post vaccination by the heat- and formalin-inactivated bacterins. The authors demonstrated, for the first time, that inactivation methods (e.g. heat vs. UV light) are fundamental in eliciting differential immune-related biological responses and discussed about the importance of vaccine formulation type (e.g. DNA vs bacterin) and administration route invasiveness (e.g bath vs. injection).

Despite not strictly focused on a Mediterranean cultured species, worth of note is a paper reporting about the construction of a vaccine based on live attenuated Phdp cells. This is in theory the most promising option because of the ability to invade the host, persist in the tissues and more effectively induce the activation of the cellular immune response [106]. A nutritionally-attenuated pathogen with a dysfunctional shikimate pathway was hence produced and delivered (either through injection or brief immersions) to 55 g BW hybrid striped bass *Morone saxatilis*. The *aroA* mutant strain proved particularly

efficient in conferring adequate protection against an immersion challenge with virulent Phdp, which caused a mean mortality of 13.3% compared to 91.65% of the non-vaccinated group [90]. Such a method would be practical for field applications, where rapidity and low dosages are required.

Among the novel tools developed for improving resistance to pasteurellosis is the use of genetic markers in genome-wide association studies (GWASs) for identifying quantitative trait loci (QTL) useful in efficient selective breeding. Palaiokostas and co-workers [107] built on previous studies [108] and improved the low resolution provided at the time by microsatellite markers by generating the first high-density linkage map for sea bream using 2b-RAD sequencing [109]. Although resolution was increased (12.085 SNPs on 24 linkage groups), the authors could not get breakthrough results because estimated heritability was low and no major statistically significant QTL for resistance was identified likely due to moderate sample size. However, the study revealed the importance of such a bioinformatic approach in potential applications to further cultured species benefitting from less developed genomic tools.

Bioinformatics was also used for *in silico* prediction of potential vaccine candidates, an approach known as reverse vaccinology [2]. The selection relied on several computational tools investigating protein subcellular localization, signal peptide motifs, hydrophobic membrane regions and similarity to bacterial virulence factors. Such a pipeline yielded 48 putative antigens from a list of 370, 8 of which were produced as recombinant proteins in *E. coli* and used for antibody production. After an *in vitro* screening by inhibition adherence assays on CHSE-214 cell lines, 10 or 25 μg of a lipoprotein were IP-injected in 13.5 g BW seabass. The highest dosage conferred protective immunity to a PDP strain 249/I99 challenge, with an RPS value of 50%.

Bioinformatic modelling algorithms were employed in an elegant and innovative work aimed at gathering knowledge for more robust vaccination strategies in fish [110]. The C-ImmSim pipeline [111] modelled features of both the innate and specific immunity (e.g. phagocytosis, cytokine release, Ag presentation, antibody secretion, binding sites of B and T lymphocytes receptors, among others). With such a computational framework it was possible to optimize administered dose and compare *in silico* and *in vivo* immunological parameters as well as fish survival following bacterial challenges. Further efforts should be made to reduce the disparity between model predictions and *in vivo* datasets.

As for solutions that have already been marketed, two commercial vaccines produced by the veterinary pharmaceutical company HIPRA entered the market in 2016, namely ICTHIOVAC® VR/PD and ICTHIOVAC® PD (Table 1). They were formulated for juvenile sae bass (~15 g BW) and for sea bream and confer comprehensive protection against vibriosis and pasteurellosis or pasteurellosis alone via IP injections or short baths/immersion delivery routes, respectively.

3.5. Flexibacteriosis

Flavobacteriosis are emerging bacterial infective pathologies in the Mediterranean area caused by Myxobacteria. The main infective bacterium is the Gram-negative *Flexibacter maritimus* (also known as *Tenacibaculum maritimum*) [112,113], a filamentous, obligatory aerobic, marine mesophilic (temperature range 15–34 °C, optimum at 30 °C) and rod-shaped (0.5 μm wide and 2–30 μm long) microorganism [114]. Tenacibaculosis is also addressed as flexibacteriosis, myxobacterial disease, salt water columnaris disease, gliding bacterial disease of sea fish, eroded mouth syndrome, black patch necrosis, peduncle disease, saddleback, fin rot cotton wool disease [115]. The disease affects a large number of marine fish species causing mortalities in aquaculture as high as 20–30% [114]. The infection was first found to be lethal in red seabream *Pagrus major* and black sea bream *Acanthopagrus schlegeli* in Japan [116]. The etiological agent was also isolated from red seabream [117] and the species was then validated under the name *Flexibacter maritimus* [118]; on the basis of phylogenetic studies, *Flexibacter maritimus* was subsequently transferred to the new genus *Tenacibaculum* as *Tenacibaculum maritimum* [119]. European cases were instead identified on Dover sole *Solea solea* first [120,121].

Analysis focusing on LPS demonstrated the existence of antigenic diversity: at least 3 major O-serogroups seemingly related to the host species have been detected. Because of a lack of strict host specificity, *T. maritimum* is able to infect a large number of feral and captive fish species worldwide such as turbot *Psetta maxima*, sole, red seabream, seabream, seabass and Atlantic salmon *Salmo salar*, just to name a few [114].

The pathology has gross external signs consisting in haemorrhagic eroded mouth, ulcerative skin lesions, hemorrhagic and necrotic lesions on the skin, fins and tail, gills, frayed fins and tail rots [114,115,122]. Both adult and juveniles fish are affected, although fish above 100 g BW appear to be more resistant. High temperatures (above 15 °C) and high water salinities (30–35‰) increase the occurrence and the severity of the disease. Environmental factors (e.g. excess of UV light), management conditions (e.g. high culture density, poor feeding) and host-related factors (e.g. body surface condition) also can increase outbreaks frequencies [114].

It is well known that the primary sites of infection are body surfaces such as the head, mouth, fins, and flanks, whereas related molecular pathways were not yet highlighted [114]. *T. maritimum* possesses many virulence factors: adhesion structures to fish mucus and hydrophobic surfaces, hemoagglutination, exopolysaccharides, adhesins, hemolysins, proteases, glycoside hydrolases, the type IX secretion system, iron uptake systems. They are likely involved in the infection process including invasion, colonization, destruction of host tissues, immune escape and nutrient scavenging [123].

Infections can be diagnosed with biochemical (isolates profile), molecular (PCR from skin/body samples) and serological methods (fluorescent antibody-based techniques) [122,124].

3.6. Vaccines and immune protection against Flexibacter maritimus

Several vaccine formulations have been tested against the reported pathogenic organisms but only a very limited number of registered vaccines is available, despite a potential high request.

The main target species of tenacibaculosis is the turbot *Psetta maxima*. Little is known about the biotic/abiotic parameters contributing to flexibacteriosis outbreaks as well as the pathogenesis itself (i.e. transmission route, lesions and immune response associated). So far, two papers have described the modulation of peripheral blood leukocytes and tissutal distribution of Ig^+ cells abundances in response to experimental *T. maritimum* infections [125,126]. A registered vaccine formulation against tenacibaculosis composed by inactivated bacteria is now available. It reportedly confers a protection higher than 75% and lasts for 6 months (Table 1), even though data from scientific literature is still lacking.

4. Conclusions

In the European Mediterranean basin more than 500.000 tons of farmed fish are produced, with seabass and sea bream representing more than 80% of the total [1]. Other species of increasing importance are the turbot, while the meagre *Argyrosomus regius*, shi drum *Umbrina cirrosa* and greater amberjack *Seriola dumerili* have only recently been introduced in aquaculture practices. So far, some efficient licensed vaccines are available for seabass and sea bream.

The great majority of commercially-available vaccines confers protection against bacterial pathologies provoked by Gram-negative bacteria such as *Listonella anguillarum*, *Photobacterium damselae*, *Streptococcus* spp., *Tenacibaculum maritimum*. These vaccines all have whole inactivated pathogens as main antigen, and the use of isolated bacterial components as active antigens is marginally reported [2].

Whole inactivated pathogens as vaccines represent a complex antigenic mixture and have a value in terms of cost/effectiveness; however, they may induce a limited protection time-wise as short as 5 weeks (Table 1).

The virus that drawed the most attention for its severity in Mediterranean aquaculture is nodavirus, and many attempts have been made to investigate its effects on the immune system of target species, mainly seabass and turbot, with the aim of producing an efficient vaccine [29–32,53,54]. A licensed vaccine against nodavirus (RGNNV strain) has been recently released, thus closing the gap (Table 1).

With the exclusion of a vaccine against *Listonella anguillarum* intended for oral administration, for which duration of stimulated immunity have not been detailed (Table 1), all other licensed vaccines are intended for administration through intraperitoneal injection or by immersion. The IP route is by far the most effective in inducing immune responses, but presents major limitations that cannot be ignored: they are the fish size, which must be sufficiently large to allow for individual injection, and the stress that may result from invasiveness of the practice. Because of these reasons, IP is unfeasible on larvae and juvenile fish.

Mediterranean farmed fish species are carnivorous predators, thus sensible to stressing conditions, and immersion/oral vaccinations should therefore be a preferential route. The mucosal route for vaccination through immersion/oral is certainly a target for future vaccination strategies, but little knowledge is presently available on this, despite its role in antigen uptake was evidenced [127–132]. In addition, mucosal vaccination could be easily adapted to early stages of fish, which can be very susceptible to pathogens, as in seabass [133]. Much work is still needed to fill the gap between the available knowledge in immune defences and the poor availability of mucosal vaccines, and it is likely that a combination of immersion priming and oral boosting will be effective.

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