New species for EU aquaculture

Deliverable Report

Deliverable No:	D25.7	**Delivery Month:**	59
Deliverable Title	Report of the major bacterial and viral diseases found in Greater amberjack, and where useful treatments have been developed, complete protocols for their implementation by the industry will be provided.		
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Task No:	25.5	**Task Lead beneficiary:**	P2. FCPCT
Task Title:	Description, diagnosis and treatment of other bacterial/viral infectious diseases occurring in amberjack		
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Objective: Report of the major bacterial and viral diseases found in Greater Amberjack, and where useful treatments have been developed, complete protocols for their implementation by the industry will be provided: a compilation will be done of the potential bacterial/viral diseases in greater amberjack related with annual seasonality. Diagnosis tools of the different bacterial/viral diseases of natural occurrence in the greater amberjack will be presented. In addition, the deliverable will include a detailed description of the symptoms found after challenge test against the most common pathogens, as a tool for diagnosis of those diseases. All those results are compiled as a diagnosis manual for the major bacterial and viral diseases found in greater amberjack and protocols for the implementation of useful treatments.

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1. Introduction

Within the last years, a compendium of the fish aquaculture diseases in Spain has been established considering the different geographical regions and the different species cultivated within each region. This classification of the different relevant pathologies was done with regard to the potential impact for the aquaculture sector, and the relevance of the different pathogens (Richard and Furones, 2012). Canary Islands has been reported to have lower incidence of fish pathogens than other regions from the Spanish mainland, highlighting the incidence of opportunistic bacteria such as *Photobacterium piscicida* and *Vibrio spp.*, and the occurrence of nodavirus in European sea bass (*Dicentrarchus labrax*). Greater amberjack (*Seriola dumerli*) has been shown to be especially susceptible to ectoparasite infections, this species having few studies on the incidence of the different pathogens cited above and was not included in the studies from the anary Islands in the above report.

2. Major bacterial and viral diseases found in Gran Canaria within the project period.

Within the different years, only few disease incidences were detected in greater amberjack and are listed in **Table 1**. All samples were seeded in BHIB supplemented with 1.5% NaCl at 25 ºC or in blood agar base (BAB, Cultimed) supplemented with 5% sheep blood and 1.5% NaCl. The bacteria grown were subjected to taxonomic analysis by standard morphological, physiological and biochemical plate and tube tests. For final identification, strains were sent to the Spanish Type Culture Collection for sequencing of 16S RNA.

Table 1. Occurrence of different pathogens within the project period.

Year	Pathogen species	Outbreaks	
2014	✓ *Vibrio harveyi* ✓ *Staphylococcus epidermidis*	October 2014 Associated with skin ulceras after monogenean infection	mortality of some juveniles 250g body weight
2015	✓ *Vibrio harveyi* ✓ *Bacillus oceanisediminis*	September 2015 Associated with skin ulceras after monogenean infection	mortality of some juveniles 400g body weight
	✓ *Photobacterium damselae* subsp. *piscicida*	October 2015	mass mortality of early juveniles (2g)
2016	✓ *Aeromonas spp.*	September 2016	Routine isolation
	✓ *V. harveyi* ✓ *V. algynolyticus*	November 2016 Associated with skin ulceras after monogenean infection	mortality occurrence in 100g juveniles
	✓ *V. algynolyticus*	December 2016 Associated with skin ulceras after monogenean infection	mortality of 500g juveniles
	✓ *Photobacterium damselae subsp. piscicida*	November 2016	mortality of early juveniles (2g)
2017	✓ *V. algynolyticus*	No outbreaks	routine isolation
	✓ *V. algynolyticus*	June 2017	1 broodstock died (15kg)
	✓ *Photobacterium damselae subsp. piscicida*	November 2017	mortality of early juveniles (6g)
2018	✓ *Photobacterium damselae* subsp. *piscicida*	August 2018	massive mortality in fry (0.2 g body weight)

An important occurrence of bacterial infections in amberjack occurs after *Neobenedenia girellae* oncomiracidia infection. When infection starts, no symptoms of secondary infection were detected in greater amberjack. However, when the parasites develop to the adult stage, 8 days after hatching (Hirazawa et al., 2010), scratching behaviour starts. As previously defined for other ectoparasite species like sea lice in Atlantic salmon (Pittman et al., 2013) and by *N. girellae* (Hirayama et al., 2009), the cranial region is considered one of the first regions were *N. girellae* attaches. For that reason, the scratching behaviour of greater amberjack starts above the eyes, were the first wounds from abrasive source appear and because of the continuous scratching, finally develop into ulcerative processes (**Fig. 1**).

Figure 1. First ulcers in the infection process of *N. girellae* in greater amberjack.

Next steps of the infection take place when the parasites move or fix to the rest of the fish, like the ventral region, where the scratches lead to wider abrasive ulcerative processes (**Fig. 2**)

Figure 2. Dorso-ventral abrasive ulcerations produced by greater amberjack scratching

The last steps of *N. girellae* infection occur with massive ulcerative processes, epithelium thickness (Hirazawa et al., 2016) and de-epithelialization (**Figs. 3&4**). Due to this physiological and immunological effort of the fish, breaches in immunocompetence and physiological status takes place, allowing emerging secondary infections by opportunistic bacteria. The main pathologies are related with *Vibrio sp*., *Flavobacterium sp., Pseudomonas p., Photobacterium damselae,* and *Streptococcus dysgalactiae* (Alcaide, 2003; Kijima-tanaka et al., 2007; Hagiwara et al., 2011). At this moment of infection, the mortalities associated to this pathology are really high, and a combination of treatments with formalin and antibiotics should be conducted.

Figure 3. Ulcerations caused by the parasite and bacterial secondary infections in the cranial region due to scratching behaviour.

Figure 4. Last step of *N. girellae* infection in greater amberjack: ulcerations caused by the parasite and bacterial secondary infections

A study on the bacteria incidence with stress associated to *N. girelliae* infection was conducted. This study consists of a stress test with selected diets assayed in Tasks 25.2. (Deliverable 25.5) and samples of liver and spleen were obtained for analysis of associated bacteria cultured.

For that purpose, 72 fish (123.18 ± 19.2 g) were randomly distributed in six 500 L tanks with twelve animals in each. Two experimental diets were challenged by triplicate, diet C (*Seriola* base diet as control) and a diet supplemented with an immunostimulant cMOS diet (concentrated MOS, ACTIGEN, developed by ALLTECH). Animals were feed three times per day to apparent satiety.

Sampling procedures

Every 30 days, growth data was collected, until the final sampling at 90 days. At this time, a stress challenge test was conducted by confinement in small cages for 5 days, taking samples in BHI of liver and spleen for bacteria cultures at the start and end of the stress challenge. Samples were incubated for 26 h at 37 ºC, and bacterial colonies obtained were isolated and identified by biochemical analyses and API 20E.

Results

No differences were obtained for final weight and for specific growth rate (SGR) after 90 days of the trial (**Fig. 5**).

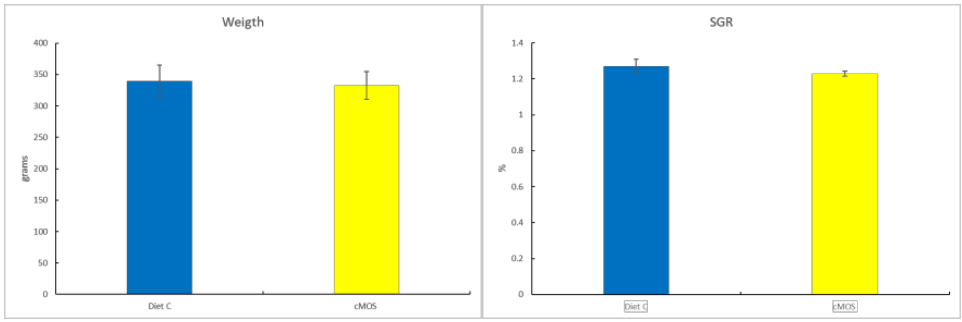

Figure 5. Final weight and specific growth rate (SGR) after 90 days of the trial. No differences were recorded (p>0.05).

Stress challenge test showed differences in prevalence of bacterial colonies in spleen and liver. At the start of the challenge, only 1 fish from 9 was positive for colonies of *Vibrio sp.*1, meanwhile no colonies were isolated for cMOS diet (**Table 2**). After 5 days of confinement, both diets showed 100% of prevalence for opportunistic bacterial colonies in spleen and liver.

Table 2. Prevalence (%) of opportunistic bacteria detected in samples from liver and spleen. Three animals per tank, a total of nine per treatment, were sampled

	T0		5 DAYS	
	Liver	Spleen	Liver	Spleen
Diet C	1/9 (11%)	1/9 (11%)	9/9 (100%)	9/9 (100%)
cMOS	0/9 (0%)	0/9 (0%)	9/9 (100%)	9/9 (100%)

Differences in the quantity of species were also detected in the bacterial cultures, with 2 different colonies of *Vibrio sp.* in cMOS diet and 4 different colonies in control diet obtained (**Table 3**)

Table 3. Different species detected in the 5 days BHI cultures of liver and spleen. *Vibrio sp.1* is related with the complex *V. anguillarum, V. damselae, V. splendidus*; *Vibrio sp.* 2 is related with the complex *V. ordalli* and *V. pelagius. Vibrio sp* 3 is related with the complex *V. vulnificus, V. argynolyticus, V. harveyi*

T0		5 DAYS	
Diet C	cMOS	Diet C	cMOS
Vibrio sp. 1		*Flavobacterium sp.*	*Vibrio sp.1*
		Vibrio sp.1	*Vibrio sp.2*
		Vibrio sp.2	
		Vibrio sp.3	

Prevalence of bacteria associated with stress was higher in fish fed the diet not supplemented with immunostimulant, whereas the addition of dietary cMOS decreased the number and amount of bacteria isolated from spleen and liver of stressed fish.

Regarding Nodavirus, 3 routine samples per year were conducted within the P2 facilities, and Nodavirus determination on amberjack broodstock within facilities was also conducted, with negative records of Nodavirus in the whole population.

For Nodavirus detection in the amberjack population PCR was used using Nodavirus (NODA) primers (**Table 4)** at a concentration 10 µM (Invitrogen).

Table 4. Primers used to detect Nodavirus. (Bp = base pairs).

PRIMERS	SEQUENCE (5'-3')	FRAGMENT SIZE
NODA 1 reverse	CGAGTCAACACGGGTGAAGA	427 bp
NODA 2 forward	CGTGTCAGTCATGTGTCGCT	

Amplification was performed in 25 µl volume containing 5 µl cDNA, 2.5 µl 10X buffer, 0.5 µl of mixture dinucleotide phosphate (20mM) (Bioron), 0.75 µl of $MgCl_2$, 1 µl of each primer, 0.125 µl Taq polymerase (Bioline) and the rest DEPC water to a total volume of 25 µl for each reaction. The protocol followed is described in **Table 5**.

Table 5. Protocol cycles for the second phase.

TEMPERATURE ºC	TIME	Nº OF CICLES
94	4'30''	1
94	30''	35
58	30''	
68	30''	
68	10'	1

The PCR products were stored at 4 ° C or -20 ° C until display in 2% agarose gel electrophoresis. A Nested-PCR for this virus was applied to the PCR products. To this end the pair of internal primers that amplify the PCR product of primers obtained from Table 1 and which generate a product of 159 bp amplification (**Table 6**) was used, at a concentration of 50 mM (Invitrogen).

Table 6. Primers used for a Nested-PCR to detect Nodavirus. (Bp = base pairs).

PRIMERS	SEQUENCE (5'-3')	FRAGMENT SIZE
R31 reverse	AGTGTCTCCAGCTTTCTTCT	159 Bp
F21 forward	GATTTCGTTCCATTCTCTTG	

Amplification was performed in 25 µl volume, containing 3 µl of Nodavirus PCR product, 2.5 ml µl of 10X buffer, 0.5 µl of mixture dinucleotide phosphate (20mM) (Bioron), 0.75 µl of $MgCl_2$, 1 µl l of each primer, 0.125 ml µl of Taq polymerase (Bioline) and the rest of DEPC water to a total volume of 25 µl, per reaction.

The results obtained are shown in **Fig. 6**: No nodavirus was found in the FCPCT greater amberjack population.

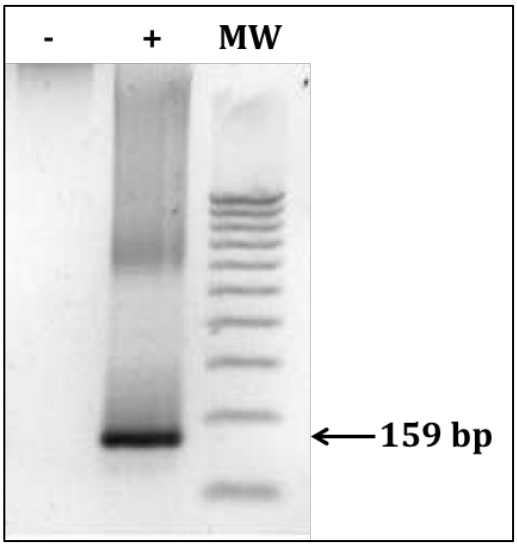

Figure 6. Amplification of Nodavirus PCR product.
Positive control for 159 base pairs.

Samples of different animals from the FCPCT facilities were analysed. From broodstock, samples from the nervous system from those broods dead during the project and frozen for analysis were analysed. From juveniles, samples of nervous system from previously stressed animals (450 g body weight) were taken after sacrifice with anaesthetic overdose. The results show a population of virus-free broodstock and a population of juveniles with no presence of virus.

3. Experimental challenge tests

Taking into account the outbreaks/occurrence of pathogens seen, different challenge tests were conducted at the Marine Biosecurity Station from P2. FCPCT.

Challenge test against Photobacterium damselae and Vibrio anguillarum.

Fifty greater amberjack juveniles (average 90 g body weight) obtained from FCPCT fish stock, were maintained in a closed seawater flow circuit with water at a temperature of 22ºC and a salinity of 37‰ under a 12-h light/12-h dark cycle. The fish were inoculated intraperitoneally with 100 µl of the different bacterial species - *Photobacterium damselae subsp. piscicida* ME-1 or *Vibrio anguillarum* at 10^5 colony-forming units/fish. Those bacteria were isolated from greater amberjack individuals. Inoculated and control fish were monitored for clinical disease and mortalities for the duration of the study (15 days). Mortality was attributed to the inoculated bacterium if the injected organism was recovered in pure culture from the internal organs (**Fig. 7**).

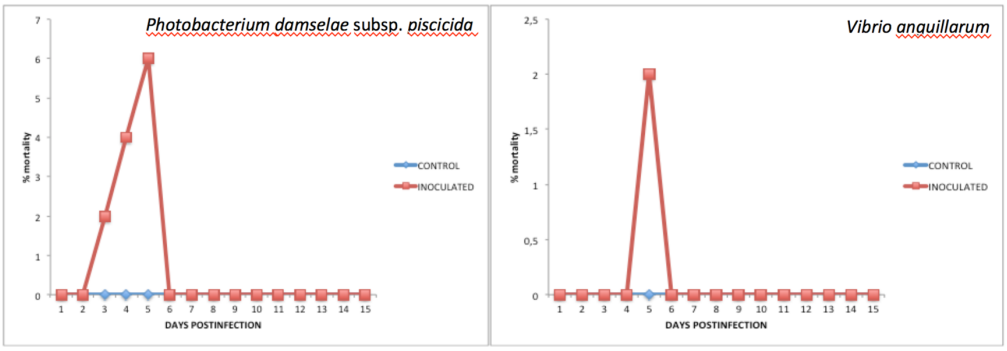

Figure 7. Results of challenge test against different bacteria isolated from individuals from the Canary Islands.

4. Minimum inhibitory concentration (MIC) of different antibiotics for the major bacteria in greater amberjack.

Florfenicol, Erythromycin, Sarafloxacin, and Oxytetracycline (Sigma-Aldrich) were the tested antibiotics. The initial concentrations used ranged from 1280µg/ml to 10µg/ml for Erythromycin, Sarafloxacin, from 800µg/ml to 6.25µg/ml, and Oxytetracylcine, from 1000µg/ml to 7.8125µg/ml, in serial dilutions with a dilution factor of 1:2. The antibiotics were diluted in TSB when used for *Vibrio anguillarum*, *Bacillus sp*., *Vibrio alginolyticus* and *Photobacterium damselae subsp. piscicida*. The total volume used for the incubation in the microtiter plate was 100µl, 50µl from bacteria and 50µl from the antibiotic solution. The experiment was repeated x3 for every bacteria and antibiotic. The turbidity of the suspensions on the microtiter plate was measured by spectrophotometry. The measurements were made at a wavelength of 600nm.

Results of MIC for *Vibrio anguillarum* (**Fig. 8**) showed that the isolated strain from greater amberjack presents MICs for erythromycin of 80µg/ml, for florfenicol of 1000 µg/ml, for oxytetracycline of 250 µg/ml and for sarafloxacin of 156 µg/ml.

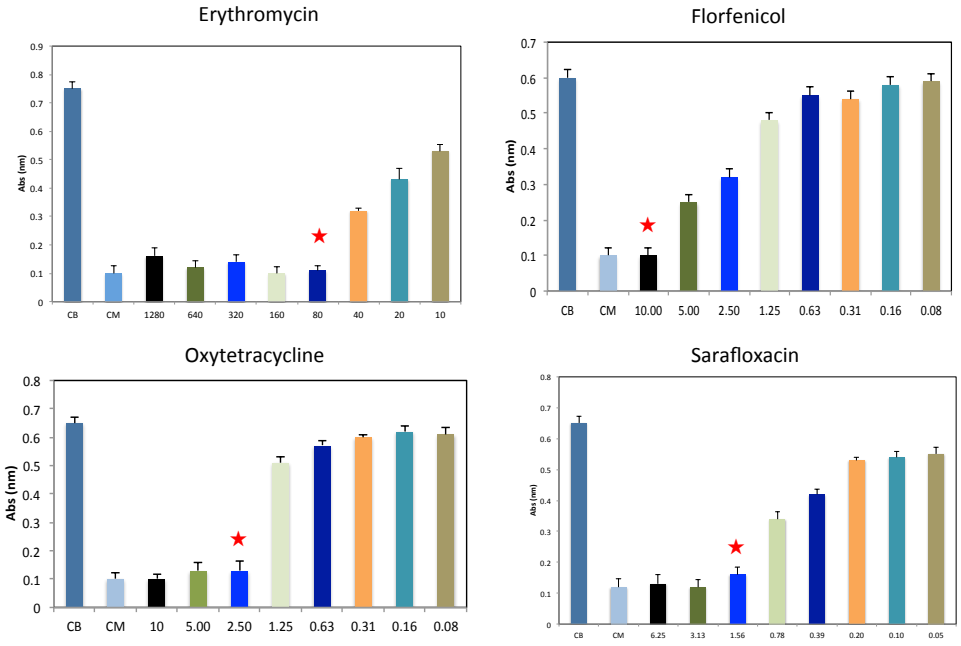

Figure 8. Absorbance obtained with respect to the concentration of antibiotic used for *Vibrio anguillarum*. The samples marked with the star represents the MIC for the tested antibiotic.

Results of MIC for *Bacillus sp.* (**Fig. 9**) showed that the isolated strain from greater amberjack presents MICs for erythromycin of 160µg/ml, for florfenicol of 1000µg/ml, for oxytetracycline of 125µg/ml and for sarafloxacin of 156 µg/ml.

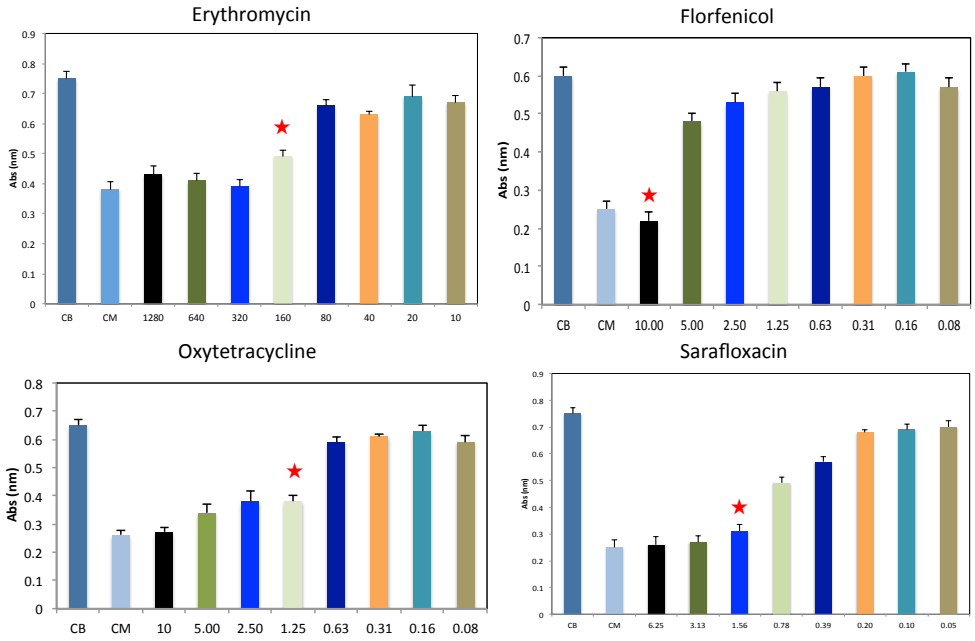

Figure 9. Absorbance obtained with respect to the concentration of antibiotic used for *Bacillum sp*. The samples marked with the star represents the MIC for the tested antibiotic

Results of MIC for *Vibrio algynolyticus* (**Fig. 10**) showed that the isolated strain from amberjack presents a MIC for oxytetracycline of 1000 µg/ml. This strain was resistant for the rest of the antibiotics tested.

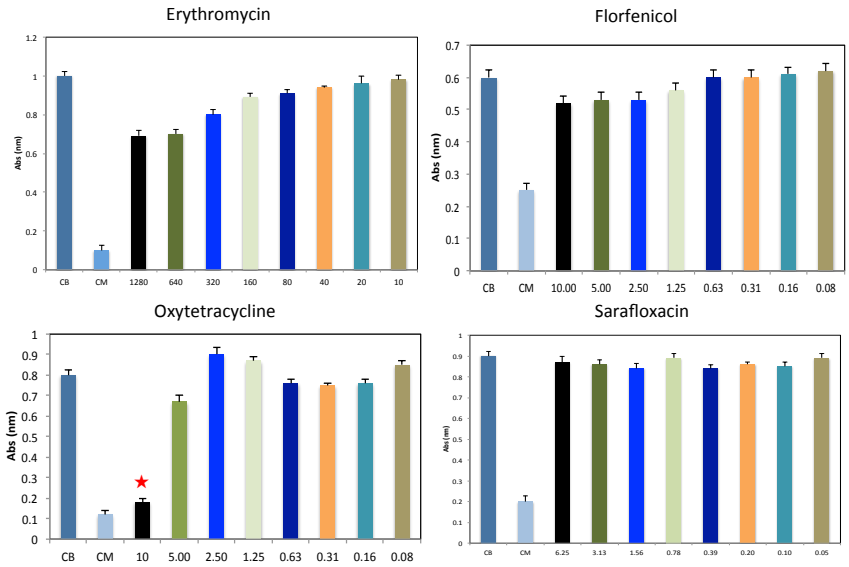

Figure 10. Absorbance obtained with respect to the concentration of antibiotic used for *Vibrio algynolyticus*. The sample marked with the star represents the MIC for the tested antibiotic.

Results of MIC for *Photobacterium damselae subsp. piscicida* (**Fig. 11**) showed that the isolated strain from greater amberjack presents MICs for erythromycin of 80µg/ml, for florfenicol of 1000 µg/ml, for oxytetracycline of 250µg/ml and the strain was resistant for sarafloxacin.

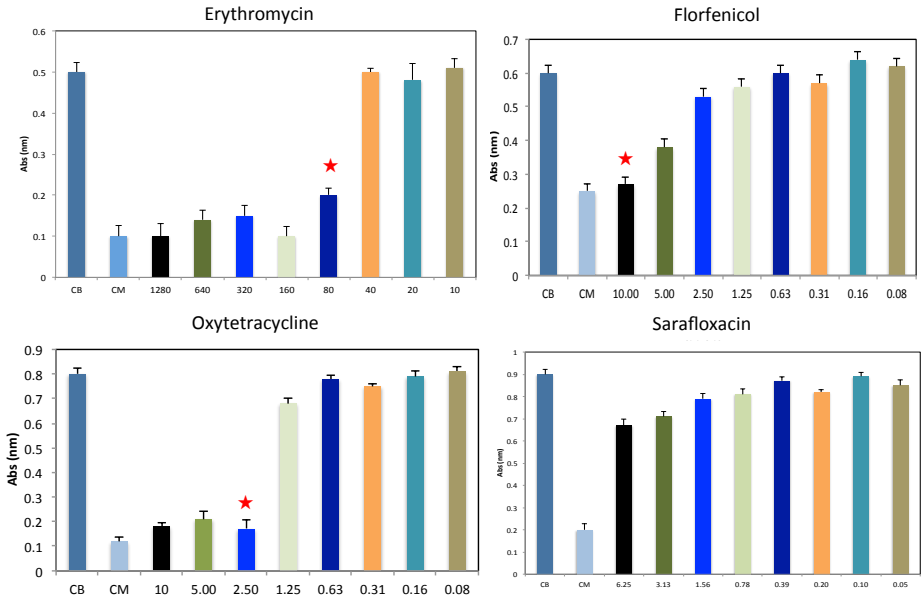

Figure 11. Absorbances obtained with respect to the concentration of antibiotic used for *Photobacterium damselae* sub. *piscicida*. The sample marked with the star represents the MIC for the tested antibiotic

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5. Recommended protocols

5.1. Molecular protocols for diagnosis by PCR

PCR And QPCR Tuning For The Diagnosis Of Pathogens

Bacterial and viral strains have been obtained from our collection. The bacteria have been grown overnight in liquid BHI at 22ºC and the viruses have been cultured in different cell lines. The DNA of the bacteria was extracted with a DNA Purification Kit (Wizard® Genomic, Promega) and the RNA of the viruses with a Total RNA Kit I (E.Z.N.A., OMEGA), following the protocol established by the manufacturer.

The pathogens that we have developed for PCR (**Fig. 12**) are:

• *Vibrio anguillarum:*

The sequences of the primers used are: VAFW 5'-ACA TCA TCC ATT TGT TAC-3 '(Forward) and VARV 5'-CCT TAT CAC TAT CCA AAT TG-3' (Reverse). These primers amplify a 429 bp fragment.
The PCR reaction is performed in a total volume of 25 µl, containing 2.5 µl 10X PCR buffer, 1.5 mM $MgCl_2$, 0.25 mM dNTP, 0.2 µM of each primer, 0.2 µg DNA, 0.5 U Taq DNA polymerase (Gen Script) and MiliQ sterile water to complete the total volume.
The amplification protocol consists of an initial denaturation at 95ºC for 10 min, 25 cycles of denaturation at 95ºC for 30 s, hybridization at 56ºC for 30 s, extension at 72ºC for 30 s, and a final extension at 72ºC for 7 min. After PCR amplification, 2 µl of the product is analyzed on a 1.5% agarose gel.

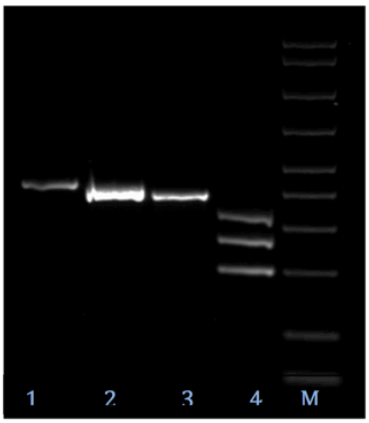
The PCR reaction is performed in a total volume of 50 µl, containing 5 µl 10X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.2 µM of each primer, 0.5 µl DNA from each Vibrio, 1 U Taq DNA polymerase (Gene Script) and sterile MiliQ water to complete the total volume.
The amplification protocol consists of an initial denaturation at 94ºC for 3 min, 30 cycles of denaturation at 94ºC for 30 s, hybridization at 60ºC for 30 s, extension at 72ºC for 120 s, and a final extension at 72ºC for 10 min. After PCR amplification, 2 µl of the product is analyzed on a 2% agarose gel.

• Multiplex of *Vibrio alginolyticus*, *Vibrio parahaemolyticus* and *Vibrio vulníficus*:

The sequences of the primers used are: VALFW 5'-GAG AAC CCG ACA GAA GCG AAG-3 '(Forward) and VALEV 5'-CCT AGT GCG GTG ATC AGT GTT G-3' (Reverse), VPFW 5 ' -GAA AGT TGA ACA TCA TCA GCA CGA-3 '(Forward) and VPRV 5'-GGT CAG AAT CAA ACG CCG-3' (Reverse) and VVFW 5'-TTC CAA CTT CAA ACC GAA CTA TGA-3 '(Forward) and VVRV 5'-ATT CCA GTC GAT GCG AAT ACG TTG-3 '(Reverse). These primers amplify a fragment of 337 bp, 271 bp and 205 bp, respectively.
The PCR reaction is performed in a total volume of 50 µl, containing 5 µl 10X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.2 µM of each primer, 0.5 µl DNA from each Vibrio, 1 U Taq DNA polymerase (Gene Script) and sterile MiliQ water to complete the total volume.
The amplification protocol consists of an initial denaturation at 94ºC for 3 min, 30 cycles of denaturation at 94ºC for 30 s, hybridization at 60ºC for 30 s, extension at 72ºC for 120 s, and a final extension at 72ºC for 10 min. After PCR amplification, 2 µl of the product is analyzed on a 2% agarose gel.

• *Photobacterium damselae subsp. piscicida:*

The sequences of the primers used are: PDSP FW 5'-AGG GGA TCC GAT TAT TAC TG-3 '(Forward) and PDSP RV 5'-TCC CAT TGA GAA GAT TTG AT-3' (Reverse). These primers amplify a fragment of 410 bp.
The PCR reaction is performed in a total volume of 25 µl, containing 2.5 µl 10X PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTP, 0.2 µM of each primer, 0.2 µg DNA, 1.25 U Taq DNA polymerase (Gen Script) and MiliQ sterile water until complete the total volume.
The amplification protocol consists of an initial denaturation at 94 ° C for 5 min, 35 cycles of denaturation at 94 ° C for 30 s, hybridization at 55 ° C for 30 s, extension at 72 ° C for 1 min, and a final extension at 72 ° C for 7 min. After PCR amplification, 2 µl of the product is analyzed on a 1.5% agarose gel.



1- *Vibrio anguillarum*. 429 pb.
2- *Photobacterium damselae* subsp. *piscicida*. 410 pb.
3- *Vibrio harveyi*. 382 pb.
4- Multiplex for *Vibrio alginolyticus* (337 pb), *Vibrio parahaemolyticus* (271 pb) & *Vibrio vulníficus* (205 pb).
M- Molecular weight marker, top to bottom:2000 pb, 1500 pb, 1000pb, 700 pb, 500 pb, 400 pb, 300 pb, 200 pb, 100 pb, 50 pb.

Figure 12. PCR for the select pathogens assayed

5.2. Recommended protocols to industry

Based on the results obtained on the occurrence of different pathogens and experimental challenge tests the following recommendations on specific antibiotic for use, dosage and protocols can be formulated. It should be noted that it is necessary to sample moribund fish and perform PCR diagnosis for the main pathogens of the greater amberjack. Depending on the result, the established treatments are:

BACTERIA	ANTIBIOTIC	DOSE	TIME	
Vibrio anguillarum	Florfenicol	10 mg/kg fish /day	Feed as the sole ration for 10 consecutive days	
	Oxytetracycline	7.5 g/100 kg fish/day	Feed as the sole ration for 5 consecutive days	**RECOMMENDED**
	Sarafloxacin	10-15 mg/kg	Single dose	
	Erythromycin	0.1 g/kg fish	Feed as the sole ration for 21 consecutive days	

BACTERIA	ANTIBIOTIC	DOSE	TIME	
Bacillus sp	Florfenicol	10 mg/kg fish /day	Feed as the sole ration for 10 consecutive days	
	Oxytetracycline	7.5 g/100 kg fish/day	Feed as the single dose for 5 consecutive days	
	Sarafloxacin	10-15 mg/kg	Single dose	**RECOMMENDED**
	Erythromycin	0.1 g/kg fish	Feed as the single dose for 21 consecutive days	

BACTERIA	ANTIBIOTIC	DOSE	TIME	
Vibrio algynolyticus	Florfenicol		RESISTANT STRAIN	
	Oxytetracycline	7.5 g/100 kg fish/day	Feed as the single dose for 5 consecutive days	**RECOMMENDED**
	Sarafloxacin		RESISTANT STRAIN	
	Erythromycin		RESISTANT STRAIN	

BACTERIA	ANTIBIOTIC	DOSE	ORAL ROUTE	
Photobacterium damselae subsp. piscicida	Florfenicol	10 mg/kg fish /day	Feed as the sole ration for 10 consecutive days	**RECOMMENDED**
	Oxytetracycline	7.5 g/100 kg fish/day	Feed as the single dose for 5 consecutive days	
	Sarafloxacin		RESISTANT STRAIN	
	Erythromycin	0.1 g/kg fish	Feed as the single dose for 21 consecutive days	

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Deviation from DOW

There was no deviation in the implemented work.

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