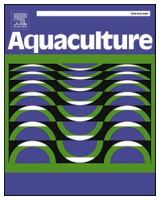
Aquaculture 581 (2024) 740458

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture



Phylogenetic reconstruction, histopathological characterization, and virulence determination of a novel fish pathogen, *Nocardia brasiliensis*

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ARTICLE INFO

Keywords:
16 s rRNA sequencing
Argyrosomus regius
Emerging disease
Granuloma
MALDI-TOF
Nocardiosis

ABSTRACT

Nocardiosis is a prevalent disease caused by various cosmopolitan aerobic actinomycetes species within the genus *Nocardia*, which has been documented across diverse aquatic species. Recently, a distinctive disease manifestation resembling classical nocardiosis emerged among cultured meagre (*Argyrosomus regius*) along the European coastline adjacent to the North Atlantic Ocean. To identify the causative agent and reproduce the disease, we conducted experiments on specific pathogen-free (SPF) meagre specimens. Employing classical methods, we isolated bacteria from liver granulomas present in multiple diseased specimens. The causative agent was subsequently identified through a combination of differential staining, MALDI-TOF mass spectrometry (MS), genus-specific nested PCR, and sequencing of the 16S ribosomal RNA gene, which facilitated the positive identification of *Nocardia brasiliensis*. The isolated strain was designated as NORBRACAN1 and considered the causative agent. Remarkably, this marks the first report of this species in fish. The pathogenicity of NORBRACAN1 was further elucidated through in vivo experimentation on a cohort of 360 juvenile meagre. The intraperitoneal administration of *NORBRACAN1* led to the development of internal microgranulomas without overt external signs or mortalities. Intriguingly, fish exposed to concentrations below 1×10^6 CFU ml^{-1} remained unaffected by mortality throughout the two-month trial period. Immunofluorescence assay unveiled the presence of viable bacteria within specific granulomas. Furthermore, our study unveiled the existence of five distinct types of microscopic granulomas characterized by necrotic centers and arranged macrophages. The formation of these granulomas was found to be contingent upon the bacterial dosage. In conclusion, our investigation demonstrates the ability of *N. brasiliensis* to colonize internal organs in marine fish and induce chronic granulomatosis. This research illuminates the potential pathogenesis of nocardiosis in aquatic species, underscoring the importance of *N. brasiliensis* as a novel causative agent in fish.

1. Introduction

Nocardiosis is an infectious disease caused by various species of the *Nocardia* genus, commonly affecting immunocompromised individuals (Rawat et al., 2022). Histopathological analysis shows *Nocardia* as a Gram-positive, branching, filamentous aerobic actinomyces with weak acid-fast, positive properties (Singh et al., 2019). *Nocardia* is widespread, found in salt and freshwater sources and various soil types, with over 80 defined species, some of which have significant implications for human disease (Rathish and Zito, 2022; Wilson, 2012). In fish, nocardiosis was initially described in sockeye salmon (*Oncorhynchus nerka)* as *Streptomyces salmonicida*. However, recent research indicates that farmed fish primarily experience nocardiosis caused by three species, namely *N. asteroides*, *N. salmonicida*, and *N. seriolae*. Additionally, *N. crassostrea* affects shellfish and is considered an opportunistic or primary pathogen (Chen et al., 2019; Elkesh et al., 2013; Han et al., 2019; Maekawa et al., 2018). Nocardiosis in both marine and freshwater fish results in chronic lesions, such as hemorrhagic ascites, hypertrophy of primary organs, granulomas, epidermal abscesses, and the formation of tubercles in the gill, kidney, and spleen (Pavloudi et al., 2023). Detailed reports are available for those interested in a more in-depth exploration of affected teleost fish species caused by *N. asteroides*,

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https://doi.org/10.1016/j.aquaculture.2023.740458
Received 14 August 2023; Received in revised form 1 December 2023; Accepted 4 December 2023
Available online 6 December 2023

N. salmonicida, and *N. seriolae* (Maekawa et al., 2018).

Distinguishing among different *Nocardia* strains can be challenging due to technical limitations in microbiology, variations in organ involvement, and the potential for mimicry with other diseases like mycobacteriosis, aspergillosis, or actinomycosis (De Benedetto et al., 2022). However, traditional microbiological methods, such as culture-based identification and differential staining, can still be used to isolate and classify *Nocardia* candidates within the genus, although this may require time. To overcome these limitations, Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) Mass Spectrometry (MS) offers a promising solution. It enables more accurate identification of microorganisms up to the species level by measuring peptides and small protein sizes on whole cells, cells lysates, or crude bacterial extracts (Chen et al., 2021). While MALDI-TOF provides high accuracy, it also faces challenges, including limited reference data sets and variability within *Nocardia* species and strains. Therefore, to complement MALDI-TOF, incorporating advanced molecular techniques is crucial (Strejcek et al., 2018). Two important methods are 16S/18S rRNA gene sequencing and the expression of the gene encoding for the heat shock protein 65 (*hsp65*). These methods have gained importance in recent years as they can help in precisely identifying different Nocardia species, given the conserved nature of the hypervariable molecular regions and the absence of horizontal gene transfer of specific genes, like *hsp65*, among different *Nocardia* species (Lei et al., 2020).

Meagre (*Argyrosomus regius*) is a highly appreciated teleost fish within the Sciaenidae family. It is found in the eastern Atlantic Ocean, ranging from the British Isles to South Africa, and can also be found in the Mediterranean and Black Seas (Zupa et al., 2020). Due to its value and potential, meagre has been proposed as a strong candidate for aquaculture diversification (Ruiz García et al., 2019). However, the successful development of meagre culture faces various complex challenges and constraints. One major concern is the risk of encountering common disease agents (Elkesh et al., 2013; Tsertou et al., 2018). Bacterial pathologies affecting meagre have been associated with *Vibrio* spp., *Photobacterium damselae* subsp. *piscicida*, and *Mycobacterium marinum* (Costa et al., 2017; Soares et al., 2018). Additionally, wild meagre specimens have been found to carry betanodaviruses of different genotypes (Lopez-Jimena and Cherif, 2010) and various monogenetic trematodes as parasites (Tedesco et al., 2022; Ternengo et al., 2010). Of particular interest is the recurrence of nocardiosis caused by *Nocardia* sp. in cultured meagre (Elkesh et al., 2013; Tsertou et al., 2018), and almost all farmed meagre populations in the Mediterranean are affected by a condition known as Systemic Granulomatosis (SG) (Pavloudi et al., 2023). Understanding the widespread occurrence of systemic granulomas is a crucial issue that demands immediate attention to facilitate the growth and expansion of the meagre aquaculture industry (Elkesh et al., 2013).

In the present study, *Nocardia* sp. was isolated in a routine sampling from the liver of apparently healthy farmed meagre in the North Atlantic area for the first time. The study aimed to investigate the phylogenetic reconstruction, histopathological characterization, and virulence of this bacterium through a combination of traditional microbiological methods and molecular assays. The results identified the bacterium as *Nocardia brasiliensis*, marking the first report of this species in fish. Furthermore, an experimental infection using *N. brasiliensis* as a novel marine fish pathogen resulted in low mortality, although significant development of severe granulomatous lesions in the liver and spleen of meagre was observed, indicating the potential pathogenicity of this bacterium in fish. These findings underscore the importance of monitoring microbial communities in aquaculture settings and raise awareness of the potential zoonotic transmission of *Nocardia* species between humans and fish. Overall, the study provides valuable insights into the presence and impact of *Nocardia brasiliensis* in the aquaculture environment and its potential as fish pathogen.

2. Material and methods

2.1. Ethical statement

All procedures involving live fish in this study were conducted in strict adherence to the guidelines of the European Union (Directive 2010/63/EU) and Spanish legislation (RD 53/2013). The study received approval from the Bioethical Committee of the University of Las Palmas de Gran Canaria (OEBA-ULPG-03/2021). Importantly, the number of animals used in this study was determined through a highly restricted sample size, calculated using a priori effect size established at a 0.05 α-error probability. To ensure statistical validity, Power analysis was performed using the GPower software (Charlie-Silva et al., 2022. By strictly adhering to ethical guidelines and conducting rigorous statistical analyses, the study prioritizes the welfare of the animals and upholds the scientific rigor of the research.

2.2. Bacterial isolates

Juvenile and adult meagre from the aquaculture facilities of the ECOAQUA University Institute in the North Atlantic region of Gran Canaria Island, Spain, were selected for the study due to the presence of an unknown pathology. To obtain samples, a number of affected fish, determined by an erratic swimming among the group were carefully removed from their enclosures and euthanized using an overdose of clove oil (López-Cánovas et al., 2020). Liver nodules from visibly diseased fish were isolated using classical microbiological methods and then streaked onto pure cultures containing brain-hearth agar (BHA) supplemented with 1.5% sodium chloride (NaCl). To further characterize the bacterial isolates, Gram staining was performed. The replicate cultures were subsequently incubated aerobically at 20 °C for 48 h (Monzón-Atienza et al., 2022). After the incubation period, the resulting bacterial isolates were preserved by freezing them at −80 °C in media supplemented with 1.5% NaCl and 20% (*v*/v) glycerol. This freezing process ensures the long-term preservation of the isolates, allowing for further analysis and investigations.

2.3. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) microorganism identification

Suggestive colonies were identified using MS analysis performed on a MALDI Biotyper® Sirius system (Brüker Daltonics, Bremen, Germany), following previously described methods (Marín et al., 2018). In brief, a fresh bacterial colony was transferred to a MALDI target spot and covered with 2 µl of matrix solution (10 mg/ml α-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 2.5% trifluoroacetic acid). To ensure MS generation reproducibility, four independent biological replicates were carried out for each culture. The obtained spectra were saved into MALDI Biotyper 3.0 software (Brüker Daltonics, Bremen, Germany) and analyzed against the first 100 peaks of each spectrum using the MBT 8468 MSP 2019 library. Quality control of the results at the species level was confirmed if two spectra had scores $\geq$2.000.

2.4. DNA isolation

Genomic DNA (gDNA) was isolated from liquid bacterial cultures using the wizard gDNA purification kit (Cat# A1125, Promega, USA). Briefly, 1 ml of the bacteria's overnight culture was added to a 1.5 ml microcentrifuge tube. The cells were centrifuged at 14,000 x*g* for 2 min, the supernatant removed, and the cells were resuspended in 480 µl of ice-cold 50 mM Tris-HCl, pH 8.0, 25% sucrose and 100 µl of lysozyme (30 mg/ml in 0.50 mM EDTA (Sigma, St. Louis, USA) and incubated for 1 h at 37 °C. Following centrifugation, 600 µl of Nuclei Lysis solution was added, cells were resuspended by pipetting, and then incubated at 80 °C for 5 min. Next, RNase A (15 mM) solution was added, tubes were inverted five times, and incubated for 30 min at 37 °C. After cooling,

200 μl of protein precipitation solution was added. Tubes were incubated for 5 min on ice, centrifuged, and the supernatant was transferred to clean tubes containing 600 μl of isopropanol. After a new round of centrifugation and drainage, the gDNA was precipitated by adding 600 μl of 70% ethanol, and the previous step was repeated. Finally, the dry pellet was rehydrated with 100 μl of DNA solution, and the tubes were incubated at 65 °C for 1 h. The resulting solution was stored at −20 °C and used later as a template DNA source.

2.5. Molecular diagnosis of Nocardia spp.

To confirm the presence of *Nocardia* spp., we employed a classical nested PCR procedure using universal 16S rRNA bacterial primers: PA-F (5′-AGAGTTTGATCCTGGCTCAG-3′) and PL06-R (5′-GCGCTCGTTGCGGGACTTAACC-3′). The initial PCR reaction yielded an expected 1000 bp amplicon. Subsequently, for the second PCR reaction, we utilized 2 μl of the previously obtained product and *Nocardia* genus-specific primers; NG1-F (5′-ACCGACCACAAGGGGG-3′) and NG2-R (5′-GGTTGTAAACCTCTTTCGA-3′). The second PCR generated a 600 bp amplicon, which was subjected to analysis using classical gel electrophoresis(Maekawa et al., 2018). Both first and second-round PCR mixtures were similar, except for using primers PA-F and PL06-R, and NG1-F and NG2-R (10 pmol/μl), in the respective reactions. In brief, each reaction was carried out in 50 μl volumes containing molecular-grade H_2O, 10× Dream Taq PCR buffer (Thermo Scientific EU, Vilnius, Lithuania), 0.5 μM of each primer, 2 mM nucleotide mixture, and Dream Taq DNA polymerase (Thermo Scientific EU, Vilnius, Lithuania). The amplification was performed using the following cycling conditions: initial denaturing at 94 °C for 15 min followed by 35 cycles of 94 °C for 45 s, annealing at 62 °C for 30 s, and extension at 72 °C for 1 min, with a final extension step at 72 °C for 10 min. Post- PCR, the quantity and quality of the amplified DNA were analyzed by spectrophotometer using an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE), and visualization was achieved through 2% agarose gel electrophoresis. Subsequently, the final PCR product underwent sequencing and was compared with known 16S rRNA gene sequences in GenBank (https://www.ncbi.nlm.nih.gov/genbank/) using the Basic Local Alignment Search Tool (BLAST).

2.6. Experimental setting and infection and challenge

To assess the pathogenicity of the recovered bacterial strain, a group of 360 meagre (*Argyrosomus regius*) with an average weight (69.5 ± 2.0 g), reared at the Marine Science and Technology Park of Universidad de las Palmas in Gran Canaria (Spain) was utilized for the study. The fish selected were specific pathogen-free (SPF) individuals, as confirmed through histological and molecular analyses conducted on 5% of the total population, showing no clinical signs of nocardiosis. These SPF fish were randomly distributed, into nine different batches of 20 individuals each in duplication, into 500 L tanks containing running seawater (maintained at 19.0 ± 0.5 °C; pH 7.2, and oxygen saturation). Throughout the experimental period, the fish were fed a commercial diet (Skretting, Spain).

To conduct the challenge, a duplicated set of eight experimental batches of fish was allocated to the treated group, while the remaining duplicated batch served as the control. The treated fish underwent intraperitoneal inoculation with 1 ml of a bacterial suspension at the exponential phase, where each concentration ranged from 10^1 to 10^8 colony-forming units per milliliter (CFU ml^{-1}). Conversely, the control group received 1 ml of sterile phosphate-buffered saline (PBS) solution. The bacterial suspension was prepared from a culture in yeast extract malt extract (YEME) broth supplemented with 0.1% Tween 80 to reduce clumping by *Nocardia* sp. The bacterial concentration was determined by performing serial plating and spectrophotometric measurements from the broth culture and its several dilutions, drawing a calibration curve. Three samplings for microbiological and histological analysis were conducted at 30-, 40-, and 50-days post-infection (dpi). At each time point, four fish per tank were randomly collected and sacrificed, and any spontaneous dead fish were recorded daily and analyzed similarly. The remaining fish were euthanized after 60 days of infection, marking the conclusion of the challenge.

2.7. Bacterial reisolate from experimentally challenged meagre

Bacteria was isolated from previously experimentally challenged meagre with *N. brasiliensis*, following the procedure described above. The isolation process involved collecting bacterial samples from the liver, spleen, and kidney. A loopful of each tissue sample was streaked directly onto three different selective media choices, namely (DSBA+1.5%NaCl, BHIA+1.5%NaCl, and YEME agar) as previously suggested (Eyler, 2013). The plates were then incubated at 20 °C for 3 weeks to facilitate the growth of bacteria, and they were checked daily for any signs of bacterial growth. Positive isolates were confirmed using both Gram stain and nested PCR techniques, as described in sections 2.2 and 2.5.

2.8. Immunofluorescent staining

Histological sections of the fixed tissues (see section 2.8) underwent permeabilization with 0.5% Triton X-100 (Sigma-Aldrich) in PBS for 24 h at 4 °C. Subsequently, the sections were blocked for 1-2 h at room temperature using a solution of 3% Bovine Serum Albumin (BSA) and 0.3% Tween-20 in PBS to minimize non-specific binding of the primary antibody. The primary antibody used in this protocol was a polyclonal rabbit anti-*Nocardia* antibody obtained following classical procedures (Harlow and Lane, 1988), with minor modifications previously described (Acosta et al., 2002). Briefly, the product was diluted at a ratio of 1:500 in 1% bovine serum albumin (BSA + 1% PBS) (Sigma), and incubated at 4 °C Prapanna et al., 2014). After incubation with the primary antibody, the tissue sections were treated with a secondary antibody, namely Alexa Fluor 488 goat anti-rabbit (Invitrogen, Cat # A-11008) at a dilution of 1:1000 dilution in PBS, for 1 h at room temperature. Following this, the sections were thoroughly rinsed in PBS five times. Finally, the slides were mounted with Fluoroshield mounting medium containing DAPI (4′,6-diamidino-2-fenilindol) from Invitrogen, facilitating nuclear staining, and observed under an Olympus BX43 epifluorescence microscopy. Digital images were acquired using an Olympus XC30 camera and merged using the Cell Sens Entry program. To ensure comprehensive analysis, at least 10 fields were randomly selected for each tissue sample, allowing for the examination of the presence of bacteria and their distribution pattern within the tissue.

2.9. Histopathology

Tissue samples of the liver, spleen, and kidney were aseptically isolated from 90 experimentally infected meagre and promptly fixed in 10% buffered formalin for 24 h. After fixation, the samples underwent routine paraffin axially embedding and were sectioned at 5 μm thickness using a microtome with an integrated water bath. Standard deparaffinization procedures were followed, and the sections were stained with hematoxylin and eosin, following the method previously described, see (Galindo-Villegas et al., 2019). Micrographs of each stained slide were captured using an Olympus CX41 microscope (Olympus Optical, PA, USA) equipped with an Olympus DP50 camera (Olympus Optical Co. LTD, Tokyo, Japan). The analysis of the images was performed using the analySIS® software package for Windows (Image Pro Plus®) provided by Media Cybernetics, Silver Spring, MD, USA. The coefficient of variation which measures the variability among individuals, was found to be <10% ensuring reliable and consistent results in the assessment.

3. Results

Following a routine health check of cultured juvenile meagers (*A. regius*), several pure bacterial isolates were obtained from the fish liver, which exhibited an obvious pathological phenotype. To ensure the purity of all liquid cultures, they were plated on BHA agar and individual specimens were re-examined. Based on the morphological and biochemical characteristics, the isolates were initially classified into general morphological groups (data not shown). At first glance, the morphological features of the bacterial isolates appeared diverse. However, these isolates were tentatively identified as *Nocardia* based on the characteristics recorded.

3.1. Nocardia isolation and morphological characterization

After cultivating a single re-isolated suspect colony for 96 h, the morphological characteristics of *Nocardia* were observed under a light microscope, confirming its branching filamentous structure and weak Gram-positive staining properties. The dry yellow bacterial colonies eventually turned white after several weeks, displaying a tangled mess of filamentous Gram-positive, branching filaments with some pink strands (Fig. 1 A, I-III). To further confirm the identity of the suspected isolates obtained from the meagers' liver, a single colony was subjected to MALDI-ToF mass spectrum (MS) analysis. The resulting peptide mass fingerprint showed a mass of approximately 7900 Da. By comparing this result with known MS spectra, the isolate was identified as *N. brasiliensis* (Fig. 1B), consistent with the previous morphological determinations.

3.2. Nocardia molecular identification

The amplification of 16S rRNA gene from our bacterial strain, tentatively identified by MALDI-ToF as *N. brasiliensis*, yielded a distinct

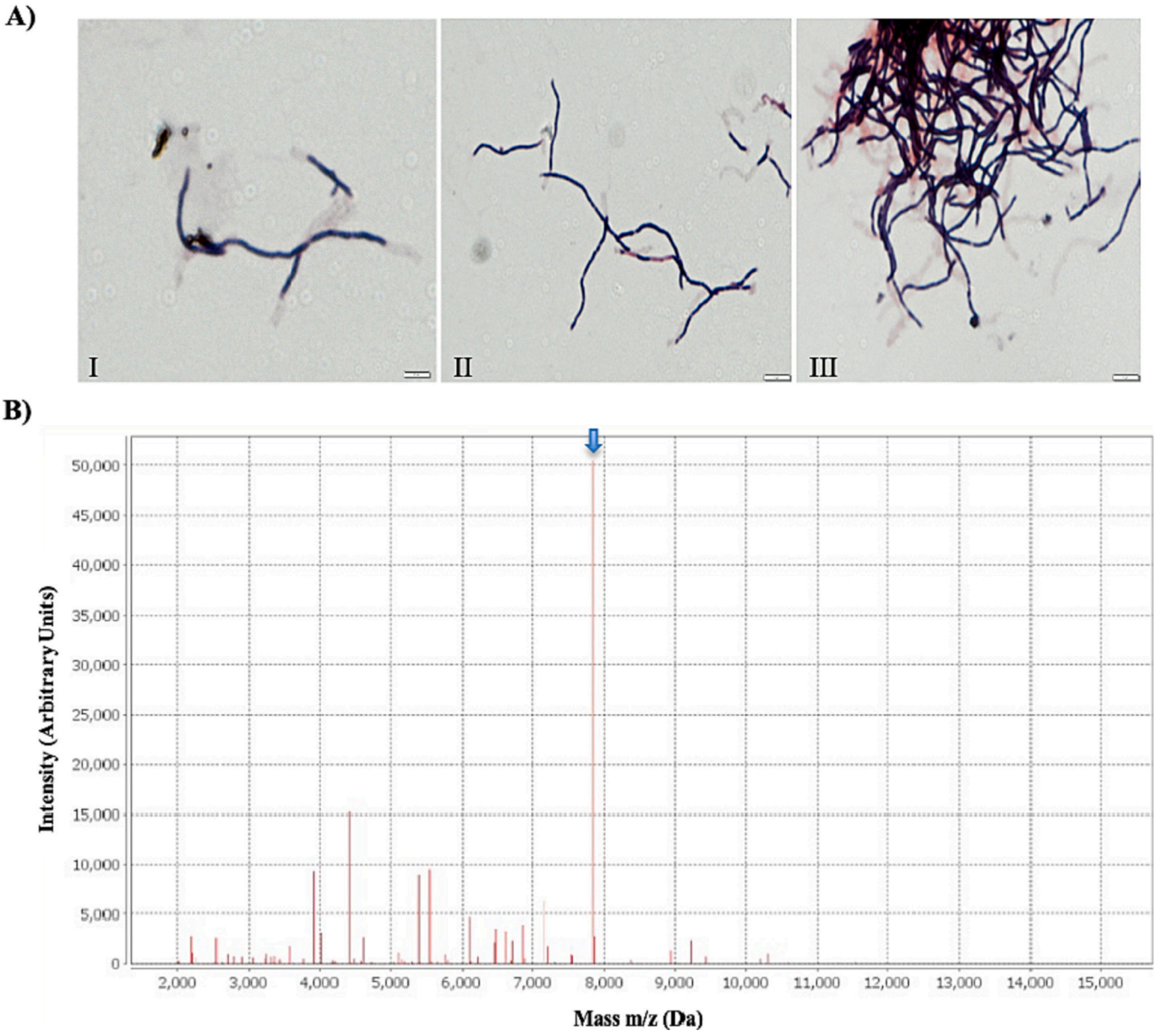


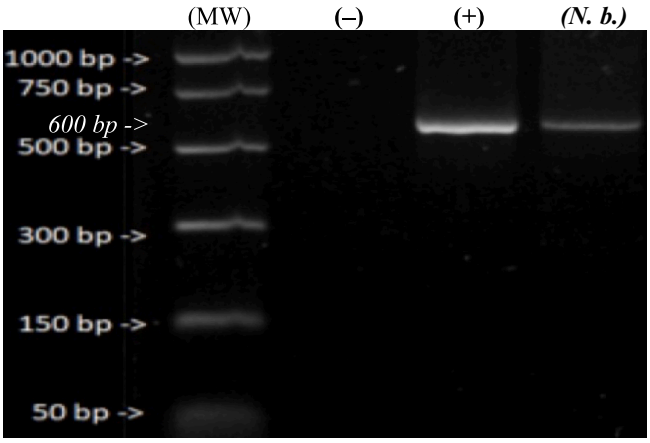
Fig. 1. Bacterial isolates obtained from cultured meagre (*Argyrosomus regius*) with an unknown pathology. A) The bacteria exhibit a characteristic *Nocardia* spp. phenotype. I-III): Showing the progression from a single colony to a tangled mess of Gram-positive bacilli. (Classical Gram stain; X400). B) MALDI-ToF mass spectrum (MS) in linear mode of the *Nocardia* isolates (Blue arrow) isolated from the liver of cultured meagre (*A. regius*). The peptide mass fingerprint (PMF) obtained was compared with known MS spectra and identified as *N. brasiliensis*.

and clear DNA band of approximately 600 bp, as shown in Fig. 2 A. This band was consistent with the expected size for the 16S rRNA gene of *Nocardia spp*, providing further confirmation of our initial identification at the genus level. To strengthen the verification of *N. brasiliensis* identity to the species level and explore its genetic relatedness with other *Nocardia* strains of the same species, we targeted a more extensive nucleotide region of the 16S rDNA for analysis. Specifically, we amplified approximately 1500-bp nucleotide fragments of *N. brasiliensis*16S rDNA using PCR, and subsequently, 838 bp of the resulting sequences were subjected to further investigation. Furthermore, the 16S rRNA gene sequence obtained from our *N. brasiliensis* isolates in this study using ClustalW in MEGA6 showed a high level of identity, over 99.88% with the type *N. brasiliensis* strain *yhl1* (MN960189.1), as represented in Fig. 2B. This finding further supports the accurate classification of our bacterial strain as *N. brasiliensis*.

3.3. Phylogenetic analysis

To establish the phylogenetic relationship, we conducted a comprehensive analysis using partial 16S rRNA sequences from various

A)



B)

Nocardia brasiliensis strain yhl1 16S ribosomal RNA gene, partial sequence

Sequence ID: MN960189.1 Length: 1458 Number of Matches: 1

Range 1: 161 to 997 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
1541 bits(834)	0.0	837/838(99%)	1/838(0%)	Plus/Plus

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Query  3    GGGTGGAAAGATTTATCGGTGCGAGATGGGCCCGCGGCCTATCAGCTTGTTGGTCGGGGT  62
Sbjct  161  ............................................................-.....  219

Query  63   AACGGCCCACCAAGGCGACGACGGGTAGCCGACCTGAGAGGGTGACCGGCCACACTGGGA  122
Sbjct  220  ............................................................  279

Query  123  CTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGG  182
Sbjct  280  ............................................................  339

Query  183  AAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCG  242
Sbjct  340  ............................................................  399

Query  243  ACAGGGACGAAGCGCAAGTGACGGTACCTGTAGAAGAAGCACCGGCCAACTACGTGCCAG  302
Sbjct  400  ............................................................  459

Query  303  CAGCCGCGGTAATACGTAGGGTGCGAGCGTTGTCCGGAATTACTGGGCGTAAAGAGCTTG  362
Sbjct  460  ............................................................  519

Query  363  TAGGCGGTTTGTCGCGTCGTCCGTGAAAACTTGGGGCTCAACCCCAAGCTTGCGGGCGAT  422
Sbjct  520  ............................................................  579

Query  423  ACGGGCAGACTTGAGTACTTCAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGC  482
Sbjct  580  ............................................................  639

Query  483  AGATATCAGGAGGAACACCGGTGGCGAAGGCGGGTCTCTGGGAAGTAACTGACGCTGAGA  542
Sbjct  640  ............................................................  699

Query  543  AGCGAAAGCGTGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTG  602
Sbjct  700  ............................................................  759

Query  603  GGTACTAGGTGTGGGTTTCCTTCCACGGGATCCGTGCCGTAGCTAACGCATTAAGTACCC  662
Sbjct  760  ............................................................  819

Query  663  CGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAG  722
Sbjct  820  ............................................................  879

Query  723  CGGCGGAGCATGTGGATTAATTCGATGCAACGCGAAGAACCTTACCTGGGTTTGACATAC  782
Sbjct  880  ............................................................  939

Query  783  ACCGGAAACCTGCAGAGATGTAGGCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGC  840
Sbjct  940  ............................................................  997
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Fig. 2. Molecular identification of *Nocardia brasiliensis*. A) Nested PCR analysis of triplicate liver extracts derived from meagre (*Argyrosomus regius*) infected with *Nocardia* spp. Both the reference strain and the extract exhibit bands at the expected size of 600 bp. MW: 100 bp DNA ladder; (−): negative control; (+): *N. asteroides* CECT 3051; (*N. b.*): recovered sample from meagre. B) Pairwise identity comparison of 16S rRNA gene partial sequence between a known *N. brasiliensis* (GenBank: MN960189.1) and the sequence isolated from meagre in this study (GenBank: OP072210).

Nocardia strains, including, *N. brasiliensis*, named from here on as NORBRACAN1 strain and utilized the BLAST algorithm available through the National Center for Biotechnology Information (NCBI) database. The resulting phylogenetic tree, generated using the neighbor-joining method, clearly indicated the close relationship between NORBRACAN1 (reported as lcl/Query_9517), the *N. brasiliensis* strain recovered from meagre, and several other reference strains of *N. brasiliensis* (Fig. 3). The analysis and clustering of NORBRACAN1 partial sequence demonstrated similar topologies with other strains of the same species, providing robust evidence for its classification within the *N. brasiliensis* species. This analysis definitively confirmed the accurate identification of NORBRACAN1 and shed light on its genetic similarity to diverse known strains of *N. brasiliensis*.

Furthermore, in the context of the same study, microscopic examination revealed the presence of granulomas at all three sampling intervals, albeit exclusively in certain infected fish that had been inoculated with the highest concentrations (10^{6-8} CFU ml^{-1}) of the pathogen. Upon aggregating data from the three samplings at 30-, 40, and − 50 dpi, and considering the three highest concentrations for each organ, it became evident that the kidney suffered the most pronounced impact, followed by the liver and the spleen, with recorded incidences of 18, 14, and 8 microgranulomas (Not observed with naked eyes), respectively.

3.4. Histopathological characteristics of N. brasiliensis

We conducted a comprehensive pathological analysis using both immunofluorescent and conventional methodologies to overarch the morphological characteristics and formation patterns of granuloma in meagre affected by *N. brasiliensis*. Despite gross examination failing to reveal the macroscopic granulomatous lesions in any organ of the experimental fish, including the liver, a meticulous ultrastructural histopathological analysis revealed the existence of a pronounced granulomatosis in the liver, kidney, and spleen of infected meagre. (Figs. 4–6).

By employing immunofluorescence techniques, in stark contrast to a presumably non-infective granuloma of unknown origin (Fig. 4 A), the specimens from which *Nocardia* sp. was isolated exhibited compelling evidence of bacterial presence within specific granulomas evident in histological sections. These granulomas exhibited prominent characteristics, including necrosis, cellular debris, and the presence of distinctive beaded fluorescent bacterial aggregates at their central regions. (Fig. 4B). Notably, a spectrum of developmental stages of granulomas, varying in size, became discernible, indicating both early and more advanced phases.

The early-stage lesions exhibited an extensive accumulation of macrophages that underwent differentiation, transforming into epithelioid cells that initially delineated bacterial colonies. Furthermore, five distinctive types of granulomas were observed: aggregates of macrophages; macrophages arranged in concentric layers; granulomas with a necrotic center with layers of macrophages; granulomas with a large necrotic center and an external layer of fibrocytes; and granulomas composed of macrophages and cellular debris arranged in a concentric laminar pattern with no necrotic center. As the inflammatory process progressed, the central region of these lesions underwent necrosis, surrounded by lymphocytes (Fig. 5 A-F).

Conversely, the mature granulomas were characterized by a necrotic core containing cellular debris and bacterial aggregates. Externally, these granulomas enveloped mononuclear cells enclosed by an external multilamellar layer comprising epithelioid cells and bacterial components. Notably, the fibrous layer thickness exhibited a positive correlation with the granuloma size.

3.5. Experimental infection

To elucidate the histopathological attributes of granulomas induced

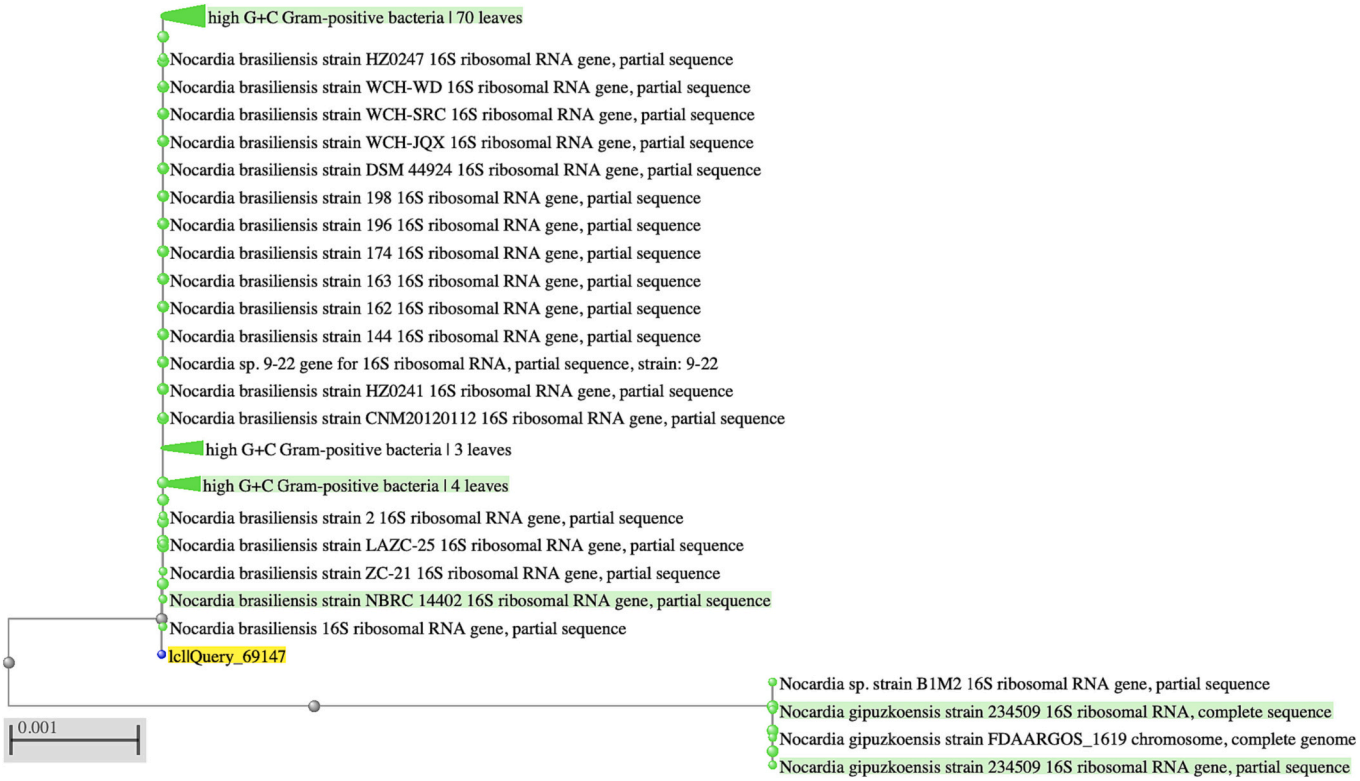

Fig. 3. Phylogenetic tree of type strains of *Nocardia brasiliensis*. The tree is constructed using the neighbor-joining method, based on partial 16S-rRNA gene sequences. It illustrates the relationships among the NORBRACAN1 clinical isolates retrieved from meagre in this study (depicted in yellow), alongside relevant phenotypical counterparts within the same species and a non-related one. Sequences highlighted in green denote type material. Gram counts (GC).

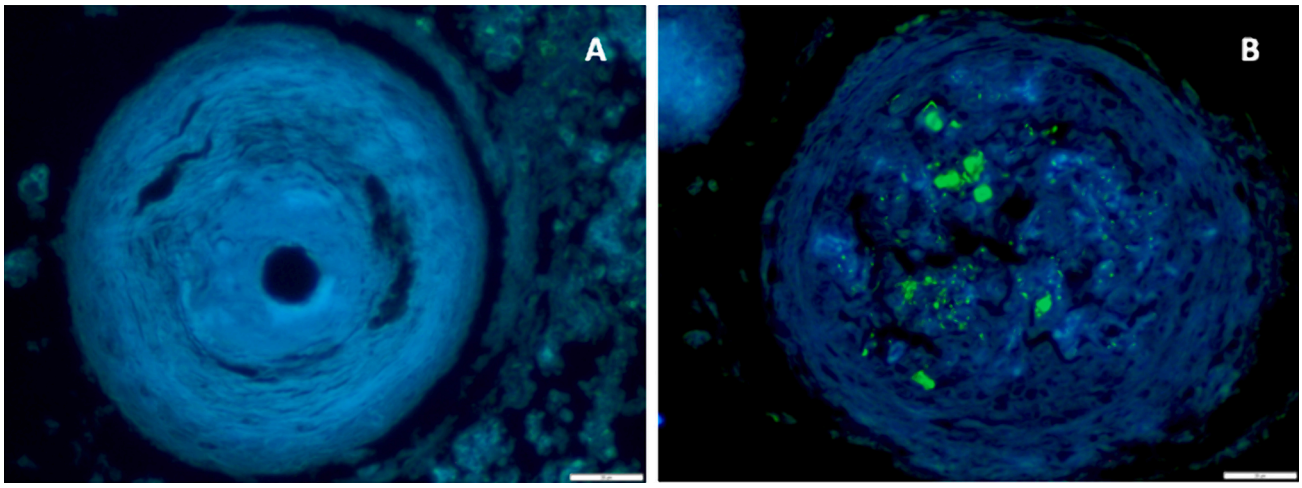

Fig. 4. Indirect immunofluorescence staining of nocardia in granulomatous inflamed liver sections of meagre. A) Non-infective granuloma. B) Positive granuloma showing nocardial aggregates in fluorescent green. Magnification: 40×. Scale bar: 20 μm.

in meagre due to the influence of NORBRACAN1, we established a model of granulomatous inflammation in meagre through intraperitoneal injection. Subsequently, the progress of infection was meticulously monitored within each group of fish by administering serial dilutions of NORBRACAN1, ranging from 10^8 to 10^1 CFU ml^{-1}, with PBS serving as the control, throughout the designated time frame. Importantly, the fish did not exhibit any sign of skin necrosis or ulceration. However, a state of lethargy was noted during the advanced phase of the challenge. Moreover, notable discrepancies in weight were observed between the groups inoculated with$10^{8–6}$ CFU ml^{-1} and those exposed to lower dosages (Data not presented). Surprisingly, during the course of the experiment, 18 out of 360 challenged fish, constituting 5% of the total individuals in the groups subjected to doses ranging from $10^{8\text{ to }6}$ CFU ml^{-1} succumbed to the infection (Fig. 6). A relatively delayed impact was evident in the group inoculated with 10^6 CFU ml^{-1}. Whitin this specific group, the progression of the infection exhibited a gradual trend over time, resulting in mortalities occurring only in the later phase of the experimental period; one fish died at 55 dpi, and one more at 57 days dpi. In contrast, an increasingly higher dosage of 10^7 CFU ml^{-1} led to a more rapid escalation of nocardiosis. Consequently, six fish succumbed to this dosage. The first death occurred at 39 dpi, followed by two more at 41 dpi, and additional two at 44 dpi, and the final one at 55 dpi. Finally, the severity of nocardiosis in fish injected with 10^8 CFU ml^{-1} exceeded the previous numbers, resulting in a noteworthy number of deaths. Specifically, five fish deaths were recorded after 39 dpi, an additional four at 41 dpi, and one more at 44 dpi. Subsequently, no further instances of mortality, whether natural or induced, were observed.

Throughout the duration of the challenge, fish belonging to the groups that were administered higher doses ($10^{8–6}$ CFU ml^{-1}) of NORBRACAN1 were subjected to random inspection for the presence of granulomas within the kidney, liver, and spleen. The granuloma architecture within the three organs displayed a similar configuration. Evidently, fibrous connective tissue was discernible in the outer layer, suggestive of a central necrotic amorphous region surrounded by a thickened multilamellar stratum comprising epithelioid cells and fibrous tissue (Fig. 7).

The emergence of these structures suggests the engagement of diverse immune processes, as indicated by the presence of various distinct leukocytes, and observed necrotic processes. Many granulomas exhibited different developmental stages, with all types being observable at any given dose and timeframe.

4. Discussion

The primary objective of this study was to explore the significant implications of *Nocardia* infection in cultured fish, with a specific and notable emphasis placed on a novel species discovery within a commercial fish culture facility. This investigation is situated within the dynamic context of meagre (*A. regius*) aquaculture operations, conducted along the European coastline adjacent to the North Atlantic Ocean. A remarkable achievement in this study is the pioneering report of *N. brasiliensis* in cultured meagre. Furthermore, the study unequivocally establishes the causal correlation between the presence of internal microgranulomas and the orchestrated infection caused by *N. brasiliensis*, all conducted meticulously within a precisely controlled experimental framework. Moreover, these distinct and discernible inflammatory aggregates underwent thorough analyses across a spectrum of temporal stages of infection progression, thereby unraveling their intricate but mild effects on the renal, hepatic, and splenic tissues.

To our knowledge, this study identifies for the first time *N. brasiliensis* as a causative strain impacting a teleost fish, specifically the meagre. In parallel, previous studies, have extensively documented nocardiosis induced by diverse *Nocardia* species, impacting both human and animal populations, particularly those with compromised immune systems (Martínez-Barricarte, 2020). In aquatic culture settings, nocardiosis has gathered significant attention (Carella et al., 2013; Hoang et al., 2020; Li et al., 2021; Teng et al., 2023; Zhang et al., 2023). Particularly, concerning cultured meagre, Elkesh et al. (2013) pioneered the initial report of systemic nocardiosis within a Mediterranean cultured meagre population, providing an exhaustive clinical description (Elkesh et al., 2013). Nevertheless, the precise species responsible for the lesions remained elusive. In a more recent study, Tsertou et al. (2018) investigated symptomatic meagre samples, positive for *Nocardia* spp., collected over a span of three years from five commercial farms and the research facilities of the Hellenic Center of Marine Research (HCMR) in Greece (Tsertou et al., 2018). Among the sampled enclosures and facilities, only fish from one farm yielded positive results for nocardiosis, with the causative agent identified as *N. seriolae*. Intriguingly, the prevailing global understanding of nocardiosis affecting teleost fish has predominantly revolved around *N. salmonicida*, and *N. seriolae* (Maekawa et al., 2018). Among these species, *N. seriolae* has emerged as the predominant pathogen, prompting the need for development of a vaccination/immunization protocol (Nayak and Nakanishi, 2016). Nevertheless, the establishment of an efficient preventive protocol demands careful optimization of variables including dosage, duration, potency, and species-specific factors (Castejón et al., 2021). However, at its core, the

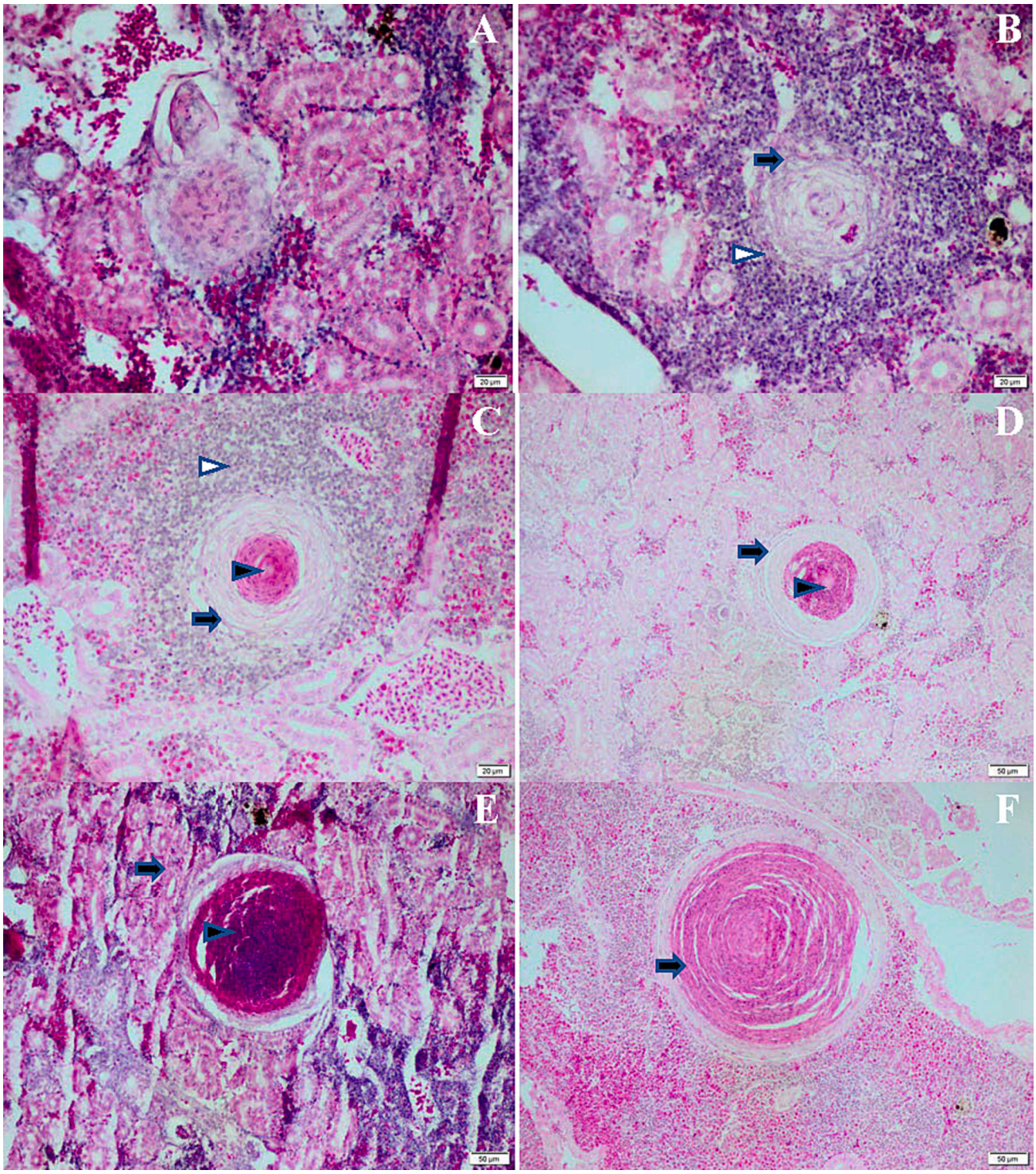

Fig. 5. Histopathological characterization of various developmental stages of liver granulomas in meagre affected by nocardiosis. A) Mature hepatic granuloma. B) Macrophages arranged in concentric layers surrounded by reactive leucocyte infiltrates (*white arrowhead*). C) Necrotic center of a granuloma (*black arrowheads*) with layers of macrophages surrounded by inflammatory infiltrates. D-E) Granulomas with a necrotic center and an external layer of fibrocytes, the granulomas center is composed of macrophages and cellular debris arranged in a concentric laminar pattern (*black arrow*). F) Granulomas without necrotic center. H&E staining. Scale bar of A, C, and E = 20 μm. Scale bar of B, D, and F = 50 μm.

foundation of a proactive prophylactic approach lies in accurately identifying the *Nocardia* species afflicting distinct fish species and the specific cultivation context.

In this study, we achieved the successful identification of *N. brasiliensis* through a multifaceted approach encompassing morphological, biochemical, microbiological, and molecular methods. Specifically, we employed nested PCRs, MALDI-TOF MS, and 16SS rRNA gene sequencing to comprehensively analyze the strain. This diverse methodological approach culminated in a robust phylogenetic analysis, based on the partial sequence of the 16 s rRNA gene among several strains of the same species, resulting in the designation of the *N. brasiliensis* found in our meagre specimens as the NORBRACAN1

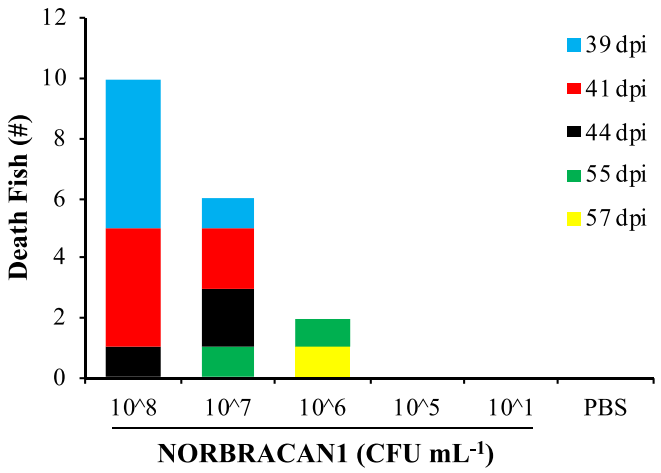

Fig. 6. Cumulative mortality of meagre (*A. regius*) experimentally infected with serial dilutions of the isolated *Nocardia brasiliensis* (GenBank: OP072210) from farmed fish. Note the low mortality recorded starting from 10^6 CFU/ml after 57 days post-inoculation.

strain. For further taxonomical comparisons, NORBRACAN1 has been deposited in the NCBI Gene Bank under the accession number MN960189.1. It is noteworthy that prior research endeavors emphasizing bacterial identification techniques have underscored the significance of adopting a combined approach involving a spectrum of distinct methodologies (Schröttner et al., 2016). Earlier investigations focused on novel constituents within the *Nocardia* genus have elucidated the inherent distinctions among species. These variances encompass diverse determinants, including selection pressures, horizontal gene transfer, functional capacity, pathogenicity, and antibiotic resistance (Xu et al., 2022a, b). Consequently, our study not only furnishes substantial insight into de unequivocal identity and presence of *N. brasiliensis* in the teleost fish species meagre but also propels advancements pertinent to the establishment of targeted preventive measures. This endeavor is facilitated through a comprehensive framework of comparative pharmacology analyses, extrapolating lessons from efficacious interventions in other vertebrate species. (Montalban-Arques et al., 2015).

Through an extensive histopathological analysis of NORBRACAN1-induced granulomatosis in meagre, this study uncovered diverse granuloma patterns linked with a mild mortality caused by *N. brasiliensis*. Notably, fish exposed to concentrations below 1×10^6 CFU ml^{-1} did not display any mortality during the two-month trial period. The absence of mortality during the established two-month challenge period underscores the need for further investigation to understand the effects of *N. brasiliensis* in meagre over an extended timeframe. Intriguingly, we observed the presence of granulomas with and without intracellular bacteria, some of which resemble those induced by *N. brasiliensis*, yet exhibited distinctive attributes. The *N. brasiliensis*-triggered granulomas featured focal aggregates of nocardial cells enveloped by infiltrating leukocytes and marked macrophage epithelioid differentiation. These findings align with prior research suggesting the action of non-pathogenic determinants, like the nutritional imbalance (not assessed in the present study) also affect cultured meagre and can be regarded as triggers for systemic granulomatous disease (Pavloudi et al., 2023; Ruiz García et al., 2019). Regarding infective granulomas induced by *N. brasiliensis*, the study identified five types of hepatic granulomas, each at different developmental stage, implying variated phases of inflammation. These characteristics were shared across granulomas triggered by most *Nocardia* spp., irrespective of fish species or developmental stage (Brosnahan et al., 2017; Lei et al., 2020; Rajme-Manzur et al., 2021; Xu et al., 2022b; Zhou et al., 2023). Surprisingly, macroscopic examinations did not reveal visible external lesions, a contrast to findings in other studies exploring *Nocardia* spp. in fish. Notably, investigations on Northern snakehead (*Channa argus*), yellowtail (*Seriola quinquerradiata*), red drum (*Scianops ocellatus*), and Asian sea bass (*Lateolabrax japonicus*) showcased skin ulcers and gill tubercules in cases attributed to systemic nocardiosis, primarily caused by *N. seriolae* (Chen et al., 2000; Del Rio-Rodriguez et al., 2021; Teng et al., 2022). This

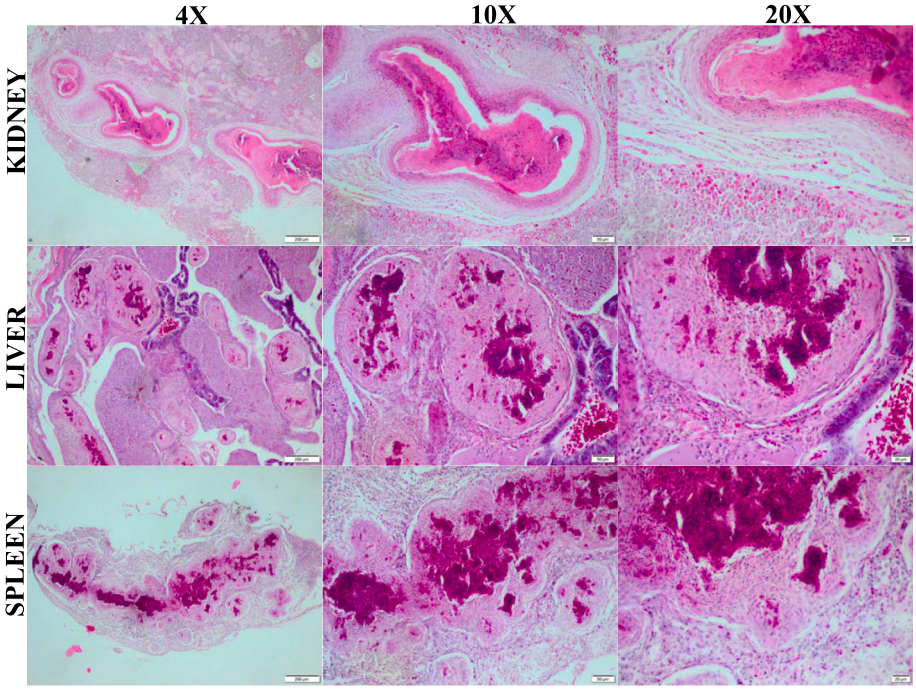

Fig. 7. Histopathological alterations in various organs of meagre following *N. brasiliensis* challenge are presented. Micrographs depict distinct irregularly shaped granulomas observed in the kidney, liver, and spleen, as revealed by classical H&E staining. These images were captured at random time-points along the 57 days trial. The extent of tissue necrosis surrounding the granulomas is prominently visible. (*N. brasiliensis* 10^{8-6} CFU ml^{-1}). Magnification; Scale bar (4×; 200 μm); (10×; 50 μm); (20×; 20 μm).

contrast underscores the distinct nature of different *Nocardia* species and highlights the relative mild impact of NORBRACAN1-induced pathology on meagre.

Overall, this study underscores the critical significance of vigilant monitoring and accurate microbial identification within aquaculture settings, particularly when dealing with emerging pathogens like *N. brasiliensis*. The research outcomes shed light on the potential repercussion of *Nocardia* infections on both fish health and the operations of aquaculture systems. These findings hold implications for effective disease management and raise concerns about potential zoonotic transmission. By elucidating the presence and consequences of *N. brasiliensis* within the aquaculture environment, this study provides valuable insights into the intricate dynamics of disease propagation and underscores the need for continued vigilance and research in this field. Nonetheless, there is a need for further exploration to comprehensively understand the complete pathogenic impact of *N. brasiliensis* in teleost fish.

CRediT authorship contribution statement

Félix Acosta: Conceptualization. **Belinda Vega:** Investigation, Methodology. **Luis Monzón-Atienza:** Formal analysis. **Joshua Superio:** Data curation, Methodology. **Silvia Torrecillas:** Data curation, Formal analysis, Methodology. **Antonio Gómez-Mercader:** Methodology. **Pedro Castro:** Methodology. **Daniel Montero:** Formal analysis. **Jorge Galindo-Villegas:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Writing – review & editing.

Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data availability

The data that support the findings of this study are available from the corresponding author upon request.

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