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First isolation of *Aeromonas salmonicida* subspecies *salmonicida* from diseased sea bass, *Dicentrarchus labrax* (L.), cultured in Spain

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ABSTRACT

This work represents the first description of *Aeromonas salmonicida* subsp. *salmonicida* as causative agent of furunculosis in cultured sea bass, *Dicentrarchus labrax* (L.). Cumulative mortality in affected fish from two floating cages in the Mediterranean coast of Spain was 3.8%. Affected sea bass did not show the typical external signs of furunculosis in the first stages of the disease, however, when the disease progressed, open ulcers appeared on the skin and muscle. Internally, splenomegaly was the only pathological sign observed. Samples from diseased fish were subjected to standardized assays for pathogens screening. Negative results were obtained for parasites and fish viruses. A Gram-stain-negative rod-shaped bacterium was observed in smears from liver, kidney and spleen of all analysed fish. Pure bacterial cultures were recovered from liver, kidney and spleen of all diseased fish sampled during the two different outbreaks. Bacteriological, serological, molecular and chemotaxonomic analysis allowed the identification of the causative agent of sea bass mortalities as *Aeromonas salmonicida* subsp. *salmonicida*. The bacterial strains were susceptible to most of antimicrobial agents usually employed in aquaculture except to oxytetracycline. Pathogenicity assays demonstrated that the isolated bacteria were virulent for sea bass, turbot and rainbow trout.

1. Introduction

Furunculosis is a bacterial fish disease caused by *Aeromonas salmonicida* subsp. *salmonicida*. This fish pathogen is one of the most studied due to its widespread distribution and the negative economic impact on wild and cultured fish (Austin and Austin, 2007; Santos et al., 2005; Toranzo et al., 2005). *A. salmonicida* mainly affects salmonid fish, producing high mortalities among Atlantic salmon, *Salmo salar* (L.) and rainbow trout, *Oncorhynchus mykiss* (Walbaum) (Austin and Austin, 2007). However, it has also been isolated from other marine and freshwater non-salmonid fish, including Atlantic cod, *Gadus morhua* (L.) (Willumsen, 1990), turbot, *Scophthalmus maximus* (L.) (Toranzo and Barja, 1992), seabream, *Sparus aurata* (L.) (Real et al., 1994), Senegalese sole, *Solea senegalensis* (Kaup) (Magariños et al., 2011), and sea lamprey, *Petromyzon marinus* (L.) (El Morabit et al., 2004). *A. salmonicida* subsp. *salmonicida* can penetrate fish through three major routes: lesions on the skin, gills as well as through the intestinal epithelia of fish (Coscelli et al., 2014; Inglis et al., 1993), disseminating throughout the tissues and causing hemorrhagic septicaemia, fin rot, soft tissue rot and the presence of the typical furuncles (Austin and Austin 2007; Bernoth et al., 1997; Coscelli et al., 2014). However, fish infected by *A. salmonicida* subsp. *salmonicida* do not always show the typical signs of the furunculosis disease (Noga, 2010). In non-salmonid fish, the pathogen manifests itself with other conditions, notably ulcerative dermatitis (Austin and Austin, 2007; Brocklebank, 1998). Moreover, several authors (Hiney et al., 1994) have reported the detection of *Aeromonas salmonicida* in Atlantic salmon with asymptomatic furunculosis infections, which may have an important role in the transmission of the disease in fish. In carrier fishes *A. salmonicida* subsp. *salmonicida* may act as an opportunistic pathogen, affecting only stressed or immunocompromised hosts (Figueras, 2005).

Sea bass, *Dicentrarchus labrax* (L.), is one of the most economically important species in the marine aquaculture sector. In the last decades, sea bass farming has significantly increased in Spain and in Mediterranean areas. As a consequence, the number of out-

Abbreviations: *A. salmonicida* subsp. *salmonicida*, *Aeromonas salmonicida* subspecies *salmonicida*; PCR, Polymerase chain reaction; MALDI-TOF, Matrix-assisted laser desorption ionization time-of-flight; TSA-1, tryptic soy agar with 1% NaCl.

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breaks of bacterial diseases has also increased. The most important bacterial diseases affecting sea bass include vibriosis, caused by *Listonella anguillarum* (Toranzo et al., 2005), and pseudotuberculosis, caused by *Photobacterium damsela* subsp. *piscicida* (Candan et al., 1996).

During May and June 2012, two separate disease outbreaks were recorded in sea bass cultured in two different floating cages in a farm located in the Mediterranean coast of Spain. Initially, affected fish did not show any external signs of disease. Internally, splenomegaly was the only pathological sign observed. When the disease progressed, open ulcers appeared on the skin and muscle. The present study describes the first isolation of *Aeromonas salmonicida* subsp. *salmonicida*, as causative agent of furunculosis infection in juvenile sea bass (*D. labrax*). Information on the biochemical, physiological, serological, molecular and chemotaxonomic characterization, virulence of the isolates, as well as susceptibility to antimicrobials commonly used in aquaculture is reported.

2. Materials and methods

2.1. Collection of fish and tissue sampling

During May and June of 2012, two separate natural outbreaks affected juveniles of *Dicentrarchus labrax* (mean ± SD; 12 ± 0.5 cm length; mean body weight 9 ± 1.0 g) cultured in two distinct sea cages of a farm located on the Mediterranean coast of Spain. Fish were stocked at a density of 0.5 kg m^{-3}. Cumulative mortality in both affected culture cages was 3.8%. Temperature during the mortality episodes fluctuated between 16 and 17 °C. Thirty diseased fish were randomly collected during each mortality episode to determine the presence or absence of fish pathogens.

Samples from brain, liver, kidney, spleen, digestive tract, gills and skin were subjected to standardized virological and parasitological analysis for diagnostic purposes. For bacteriological analysis, samples of liver, kidney and spleen were used.

2.2. Pathogens detection and identification

Fish were examined externally and internally for parasites by the diagnostic service of Skretting (Spain). Virological analysis for detection of nervous necrosis virus was carried out as previously described (Panzarin et al., 2010). Fuchsin and Gram stained smears from liver, kidney and spleen were also examined using a light microscope. For bacteriological analysis samples from liver, kidney and spleen of sea bass were cultured on BD Columbia III Agar with 5% Sheep Blood (Becton Dickinson GmbH) and incubated at 18 ± 1 °C for 24–96 h. Pure bacterial cultures were recovered on blood agar plates from all fish analysed. Two colonies recovered from each fish were transferred onto trypticase soy agar supplemented with 1% NaCl (TSA-1) and incubated at 18 ± 1 °C during 2–3 days for further characterization. In all cases, colonies producing brown diffusible pigment were recovered. Pure cultures of these isolates were stored at −30 °C in Microbank™ commercial medium (Pro-Lab Diagnostics, Ontario, Canada) until use.

Five bacterial strains isolated during each mortality episode were characterized using morphological, physiological and biochemical standard tests, and API 20E (BioMérieux, Madrid, Spain) as previously described (Santos et al., 1993). Sea bass isolates were also evaluated for their halotolerance by its growth on basal medium (4 g Neopeptone, 1 g yeast extract, 15 g agar in 1 L of distilled water), containing different concentrations of NaCl (0–10%). Susceptibility to antimicrobials was evaluated by the agar diffusion tests following the procedures of the Clinical and Laboratory Standards Institute document M42-A (CLSI, 2006). Antimicrobials agents (Oxoid and BD) and concentrations used were: ampicillin (10 μg), amoxicillin (25 μg), flumequine (30 μg), oxytetracycline (30 μg), enrofloxacin (5 μg), florfenicol (30 μg), thrimethoprim-sulfametoxazole (25 μg), oxolinic acid (2 μg) and pteridine (150 μg). The quality control strain recommended by CLSI, *Aeromonas salmonicida* subsp. *salmonicida* ATCC 33658 from the American Type Culture Collection (ATCC), was used as positive control in all the assays. Except otherwise stated all incubations were carried out at 18 ± 1 °C.

The serological characterization of the ten sea bass isolates was performed using the slide agglutination and Dot Blot assay as previously described (Santos et al., 1995). The tests were carried out using whole cell (formalin inactivated bacterial suspensions) and "O" antigens (100 °C h^{-1} treated bacterial suspensions) adjusted to contain 10^9 cells mL^{-1} and rabbit whole cell antiserum raised against the strain TO96 7.1 of *A. salmonicida* subsp. *salmonicida* produced in our laboratory as described by Santos et al. (1995).

Reference strains of *Aeromonas salmonicida* subsp. *salmonicida, A. salmonicida* subsp. *achromogenes*, *A. salmonicida* subsp. *masoucida* and *A. salmonicida* subsp. *smithia* from the ATCC, as well as *A. salmonicida* subsp. *salmonicida* isolated from different fish species were used for comparative purpose in phenotypical and serological analysis (Table 1).

2.3. Molecular characterization

DNA from pure cultures of all bacteria used in this study was obtained using InstaGene matrix (Bio-Rad, Madrid, Spain). DNA concentration was quantified using the fluorimeter Qubit® 2.0 and the Qubit® DNA BR Assay Kit system. PCR amplifications were performed using the specific primers Fer3 and Fer4 that amplified a region of 422 bp of the *fstA* gene (implicated in the functioning of the ferric siderophore receptors) and DNA obtained from bacteria (10 strains isolated from sea bass and all reference strains), following the procedure described by Beaz-Hidalgo et al. (2008). Reactions lacking DNA or including DNA from the reference strain of *A. salmonicida* subsp. *salmonicida* ATCC33658 were used as negative and positive controls, respectively. PCR products were separated on a 1% (w/v) agarose gel during 1 h at 100 V and stained with 5 μL of Redsafe nucleic acid staining solution (20.000x) (iNtRON Biotechnology). A 100–3000 bp DNA ladder (Fermentas) was included as a molecular weight marker. PCR products were visualized using an ultraviolet light transilluminator (Bio-Rad UV Transilluminator 2000). The presence of a band with a size of 422 bp was considered as a positive result.

2.4. Chemotaxonomic analysis

The fatty acid profile of all strains used in the study was evaluated in duplicate in different cultures of the same bacterium. The processes including cell harvesting, saponification of lipids, methylation of fatty acids, extraction of fatty acid methyl esters, washing of extracts and gas chromatography analysis was performed following the standardized procedures for the Microbial Identification System (MIDI; Microbial ID Inc., Newark, DE, USA) (Sasser, 2001).

Proteomic analysis of all strains used in the study was performed by MALDI-TOF mass spectrometry analysis. Briefly, a single bacterial colony was deposited onto the MALDI-TOF target plate. Subsequently, 1 μL of 70% formic acid was added and left to dry. Bacterial spot was overlaid with 1 μL of the matrix solution, containing saturated α-cyano-4-hydroxycin-namic acid (α-CHCA) (Sigma-Aldrich, St Louis, MO) in 50% acetonitrile, and was allowed to dry at room temperature. For each sample, two extractions were made, and both extracts were spotted in duplicate, to verify reproducibility. Mass spectra were generated by a Microflex MALDI-TOF-MS mass spectrometer (Bruker Daltonics, Germany),

Table 1
Sea bass isolates and reference strains of *A. salmonicida* used in this study.

Bacteria	Source	API 20E profile
Strains isolated during 1st outbreak (May 2012)		
SK164/12.1	*Dicentrarchus labrax*	6006104
SK164/12.2	*D. labrax*	6006104
SK164/12.3	*D. labrax*	6006104
SK164/12.4	*D. labrax*	6006104
SK164/12.5	*D. labrax*	6006104
Strains isolated during 2nd outbreak (June 2012)		
SK181/12.1	*D. labrax*	4006104
SK181/12.2	*D. labrax*	4006104
SK181/12.3	*D. labrax*	4006104
SK181/12.4	*D. labrax*	4006104
SK181/12.5	*D. labrax*	4006104
Reference strain		
A. salmonicida subsp. *salmonicida* TO96 7.1	*Scophthalmus maximus*	6006104
A. salmonicida subsp. *salmonicida* MT416	*Salmo salar*	2006104
A. salmonicida subsp. *salmonicida* AsV09.2.15	*Oncorhynchus mykiss*	2006104
A. salmonicida subsp. *salmonicida* ATCC33658	*S. salar*	6006104
A. salmonicida subsp. *achromogenes* ATCC33659	*Salmo trutta*	2226066
A. salmonicida subsp. *masoucida* ATCC27013	*Oncorhynchus masou*	3267166
A. salmonicida subsp. *smithia* ATCC49393	*Rutilus rutilus*	2226046

ATCC, American Type Culture Collection (Rockville, MD, USA)

equipped with a 337 nm N_2 laser. Prior to analysis, mass calibration was carried out using a Bruker IVD Bacterial Test Standard (Bruker Daltonics, Germany). The overall mass range covered by IVD BTS is 3.6–17 kDa. Mass spectra were analysed using the FlexAnalysis 3.0 software (Bruker Daltonics, Germany), baseline corrected, noise filtered, and data lists containing m/z values were extracted from mass spectral data. The peak lists generated from each strain were imported, analysed by standard pattern matching using BioTyper 3.0 software (Bruker Daltonics, GmbH) and results were expressed using the criteria proposed by the manufacturer. The identification was considered reliable at the species level, if the score value was higher than 2.

The SPECLUST software was used to process the four spectra obtained for each bacterial sample, to calculate the arithmetic means of the m/z values of the replicates and to determine the representative common peak masses. The specific mass lists (m/z) of the strains were compared with each other with the application SPECLUST in order to identify the characteristic peak masses and to define subspecies-specific biomarkers. Mass range of 2000–10,000 m/z was used for spectral analysis. Similarities were calculated using curve-based (Pearson Product Moment Correlation Coefficient, PPMCC), as well as band-based (Jaccard similarity coefficient) measures and single-linkage. For cluster analysis IBM SPSS statistics V22 was used.

Lipopolysaccharides (LPS) from all *A. salmonicida* subsp. *salmonicida* strains used in the study were obtained following the method described by Hitchcock and Brown (1983). Total membrane proteins were obtained as described (Santos et al., 1995). Protein concentration was quantified using the fluorimeter Qubit® 2.0 and the Qubit® Protein Assay Kit system, following the protocol recommended by the manufacturer (Invitrogen). The LPS and proteins were analysed by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) (Laemmli, 1970) using the Bio-Rad Mini Protean II slab cell system. Standard protein markers from Bio-Rad (Laboratories, Richmond, California) were used as controls. Proteins were stained with Comassie brilliant blue R-250 (Sigma Chemical Co., St. Louis, Mo.), and LPS were silver stained by using the Silver Stain Plus method (Bio-rad).

Immunoblot analysis of LPS and protein were performed basically as described by Towbin et al. (1979) using serum against *A. salmonicida* subsp. *salmonicida* strain TO96 7.1 and goat anti-rabbit immunoglobulin G-alkaline phosphatase conjugate (Bio-Rad) as primary and secondary antibodies. Bands were visualized after incubation of the membrane in 0.1 M carbonate buffer (pH 9.8) containing tetrazolium blue (Sigma) (0.3 mg mL^{-1}) and 5-bromo-4-chloro-3-indolylphosphate toluidine salt (BCIP) (0.15 mg mL^{-1}) (Fermentas International Inc., Ontario, Canada).

2.5. Pathogenicity assays

Fish were obtained from farms located in Galicia (Spain). The assays were carried out at the aquarium facilities of the University of Santiago de Compostela. Pathogenicity assays were carried out by intraperitoneal injection of sea bass (average weight of 7.5 ± 0.1 g), turbot (5.0 ± 0.1 g) and rainbow trout (14.5 ± 0.5 g), following the methodology described by Santos et al. (1991). Turbot and sea bass were reared in separate 500 L tanks, aerated and supplied with sand-filtered seawater at 32‰ of salinity at 16 ± 1 °C. Rainbow trout were maintained in 500 L tanks supplied with aerated freshwater at 16 ± 1 °C. Fish were fed on commercial diet during the course of the experiments. Before challenge experiment, fish were subjected to bacteriological, virological and parasitological analysis in order to verify their health status.

Two strains isolated from sea bass during the episodes of mortality that occurred in May (strain SK164/12.1) and June (strains SK181/12.1) were selected for virulence assays. Bacterial suspensions were prepared, adjusted to 10^9 cells mL^{-1} (McFarland scale tube 3) and serially ten-fold diluted using saline solution (0.9% NaCl). Colony forming units (CFU) were enumerated by the plate dilution method by seeding bacterial cell suspensions onto TSA-1 agar plates and counting the bacterial colonies produced. For challenge, fish were anaesthetised by immersion in tricaine methane sulfonate (MS-222, Sigma) (60 mg L^{-1}, Neiffer and Stamper, 2009) and intraperitoneally injected with 0.1 mL of bacterial suspensions, containing 2×10^8, 2×10^7, 2×10^6 and 2×10^5 CFU mL^{-1} (10 fish per dose). Infected fish were kept in tanks (length 115 × width 96 × depth 65 cm) on the conditions above described. Fish mortalities were considered caused by the inoculated strain, only if the bacterium was recovered in pure culture from liver, kidney and spleen of dead or dying fish or if it was detected by PCR in tissues of dead fish. DNA from kidney and spleen of dead and moribund fishes was extracted using Dynabeads DNA Direct System for the detection of *A. salmonicida* subsp. *salmonicida* by PCR. The identity of the recovered bacteria was also confirmed by serological assays.

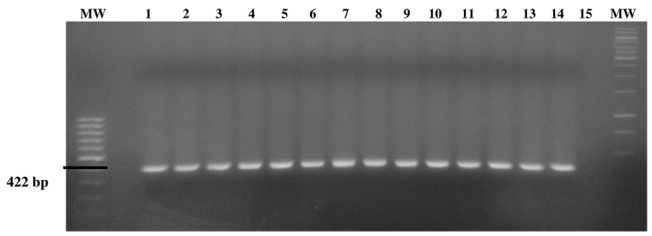

Fig. 1. PCR analysis of *A. salmonicida* subsp. *salmonicida* strains using Fer-3 and Fer-4 primers. Lanes: MW, molecular weight marker (Thermo scientific); 1, ATCC33658; 2, TO96 7.1; 3, MT416; 4, AsV09.2.15; 5, SK 164/12.1; 6, SK 164/12.2; 7, SK 164/12.3; 8, SK 164/12.4; 9, SK 164/12.5; 10, SK 181/12.1; 11, SK 181/12.2; 12, SK 181/12.3; 13, SK 181/12.4; 14, SK 181/12.5; 15, Negative control.

3. Results

3.1. Bacterial characterization

Phenotypic tests demonstrated that all the bacteria isolated from sea bass tested were Gram-stain-negative, non-motile rods, catalase and oxidase positive and fermentative. All isolates were able to grow on medium containing concentrations up to 3% NaCl, however, no growth was observed when higher concentrations of salt were used. Moreover, all the isolates from sea bass as well as the reference strains of *A. salmonicida* subsp. *salmonicida* gave the typical profiles described for *A. salmonicida* in the API-20E identification system. The most common API profiles were 6006104 (7 strains), followed by 4006104 (5 strains) (Table 1). The agar diffusion tests showed that all the isolates from sea bass were sensitive to all the antimicrobial agents used, except to oxytetracycline and the vibriostatic agent pteridine (O/129). Only two *A. salmonicida* subsp. *salmonicida* reference strains (ATCC33658 and AsV09.2.15) and all atypical subspecies were sensitive to oxytetracycline. Slide agglutination and Dot Blot assays showed that antigens from all strains isolated from sea bass and reference strains of *A. salmonicida* subsp. *salmonicida* reacted with the anti-*A. salmonicida* TO96 7.1 serum. Cross reaction was observed when the strain of *A. salmonicida* subsp. *masoucida* CECT896 was tested with anti-*A. salmonicida* TO96 7.1 serum. However, whole cells and "O" antigens of *A. salmonicida* subsp. *achromogenes* CECT895 and *A. salmonicida* subsp. *smithia* CECT5179 did not react with anti- *A. salmonicida* TO96 7.1 serum.

Evidence of viral and parasites fish pathogens were not detected in any sampled fish.

3.2. Molecular characterization

PCR reaction using primers Fer3 and Fer4 described by Beaz-Hidalgo et al. (2008) allowed us to identify the ten bacterial strains isolated from sea bass as well as the reference strains as *A. salmonicida*. Using DNA obtained from pure cultures of all *A. salmonicida* strains tested, a specific amplicon of 422 bp was detected. Fig. 1 shows the results obtained with sea bass isolates and *A. salmonicida* subsp. *salmonicida* reference strains.

3.3. Chemotaxonomic analysis

The cellular fatty acid content (%) of all sea bass isolates and reference strain of A. *salmonicida* subsp. *salmonicida* was characterized by the presence of straight chain saturated (% ranging from 31.43 to 33.75) and monounsaturated fatty acids (% ranging from 13.52 to 16.04). The major fatty acids (those present at levels higher that 1%) were C12:0, C14:0, C16:0, C17:0, C17:1ω8c, C18:1ω7c. Comparison of the fatty acid methyl esters profiles of *Aeromonas salmonicida* subsp. *salmonicida* isolates from sea bass with those of the MIDI data base allowed the correct identification of the strains at species level.

All strains isolated from sea bass were correctly identified as *A. salmonicida* subsp. *salmonicida* (score values ranging from 2.1 ± 0.07 to 2.39 ± 0.08) after analysis of the mass spectrometer protein profiles with the BioTyper 3.0 software. Peak mass, expressed as the arithmetic means of the m/z values of four replicates of each strain, were compared using the SPECLUST software. Proteomic profiles of strains SK181/12.1, SK181/12.2, SK181/12.3, SK181/12.4 and SK181/12.5 from the second outbreak in June 2012 were identical to those obtained with all the strains of the subspecies *A. salmonicida* subsp. *salmonicida* used for comparative purpose. However, bacterial isolates from the first outbreak in May 2012 (SK14/12.1, SK14/12.2, SK14/12.3, SK14/12.4, and SK14/12.5) showed a characteristic peak mass of m/z 2747.16 (Table 2). A species specific peak mass of m/z 2028.35 were observed in all *A. salmonicida* strains. Moreover, subspecies specific peak masses were found in *A. salmonicida* subsp. *achromogenes*, *A. salmonicida* subsp. *masoucida*, and *A. salmonicid*a subsp. *smithia*. In Table 2 characteristic peaks masses of one strain isolated during each outbreaks as well as one strain per bacterial subspecies of *A. salmonicida* is presented.

Cluster analysis of the protein profile, showed that all the reference strains of *A. salmonicida* subsp. *salmonicida* and the isolates from sea bass, grouped together on the basis of the similarity (88%) of their mass spectra profile (Fig. 2) and were separated from the reference strains of *A. salmonicida* subsp. *masoucida*, *A. salmonicid*a subsp. *achromogenes* and *A. salmonicida* subsp. *smithia*. Similarities calculated using PMCC and Jaccard measures resulted in comparable identification results.

The membrane protein profiles of all strains of *A. salmonicida* subsp. *salmonicida* were analysed by SDS-PAGE and immunoblot. The electrophoretic analysis showed the existence of homogeneity among all strains of *Aeromonas salmonicida* subsp. *salmonicida* used in this study, with protein bands ranging from 12 to 156 kDa (data not shown). The reference strain of *A. salmonicida* subsp. *salmonicida* ATCC33658 showed an identical pattern. The immunoblot analysis revealed that cell envelope preparations of all *A. salmonicida* subsp. *salmonicida* reacted with rabbit serum against the strain TO96 7.1 of *A. salmonicida* subsp. *salmonicida*, showing a characteristic major band of 40 kDa (data not shown).

The analysis of LPS demonstrated that all the strains used in this study showed an identical profile regardless of their isolation source. Moreover, immunoblot analysis revealed that LPS reacted with antiserum against the strain TO96 7.1 of *A. salmonicida* subsp. *salmonicida* (data not shown).

3.4. Pathogenicity assay

The virulence assay demonstrated that strains of *A. salmonicida* subsp. *salmonicida* (SK164/12.1 and SK181/12.1) were virulent for sea bass, rainbow trout and turbot, causing 100% fish mortality with all bacterial doses evaluated (2×10^7 to 2×10^4 CFU per fish). The bacterium was recovered in pure cultures from liver, kidney and spleen of all moribund and dead fish and identified as *A. salmonicida* subsp. *salmonicida* by serological methods and PCR.

4. Discussion

The present study reports for the first time an episode of furunculosis, causing mortalities in sea bass cultured in sea cages in the Mediterranean coast of Spain. The phenotypic characteristics of strains isolated from sea bass were identical to those described for *A. salmonicida* subsp. *salmonicida*. All sea bass isolates and the reference strains of *A. salmonicida* subsp. *salmonicida* evaluated,

Table 2
Characteristic peaks masses of the subspecies of *A. salmonicida*.

SK164/12.1 Bacterial isolate from 1st outbreak, (May 2012)	SK181/12.1 Bacterial isolate from 2ndoutbreak (June 2012)	*A. salmonicida* subsp. *salmonicida* ATCC33658	*A. salmonicida* subsp. *achromogenes* ATCC33659	*A. salmonicida* subsp. *masoucida* ATCC27013	*A. salmonicida* subsp. *smithia* ATCC49393
2028.35*	2028.35	2028.35	2028.35	2028.35	2028.35
2127.46	2127.46	2127.46	**2105.56**	2127.46	**2461.93**
2747.16	3240.09	3240.09	2127.46	**3011.77**	**2920.31**
3240.09	3435.62	3435.62	**3112.87**	3240.09	**3082.85**
3435.62	3595.69	3595.69	**3293.22**	**3372.66**	3240.09
3595.69	4260.10	4260.10	**3400.97**	3435.62	**3435.62**
4260.10	4371.13	4371.13	3595.69	3595.69	**3739.54**
4371.13	4595.81	4595.81	**4117.59**	**3762.02**	4260.10
4595.81	5156.84	5156.84	**5105.5**6	4260.10	4371.13
5156.84	5534.26	5534.26	6086.98	4371.13	4595.74
5534.26	6086.98	6086.98	6478.28	4595.81	5156.84
6086.98	6478.28	6478.28	**6804.55**	5156.84	5534.56
6478.28	6850.39	6850.39	6850.39	5534.26	**5930.05**
6850.39	7333.91	7333.91	7195.51	6086.76	6850.39
7333.91	8936.98	8072.35	7333.91	6477.70	**7283.06**
8936.98		8936.98	8072.35	**6747.92**	8072.35
			8884.05	7195.51	
			8936.98	7333.89	
				8072.35	
				8936.98	
				9014.04	

*Peak mass values are expressed as the arithmetic means of the *m*/*z* values of four replicates. Subspecies-specific peaks are highlighted in bold and species-specific peaks in italics.

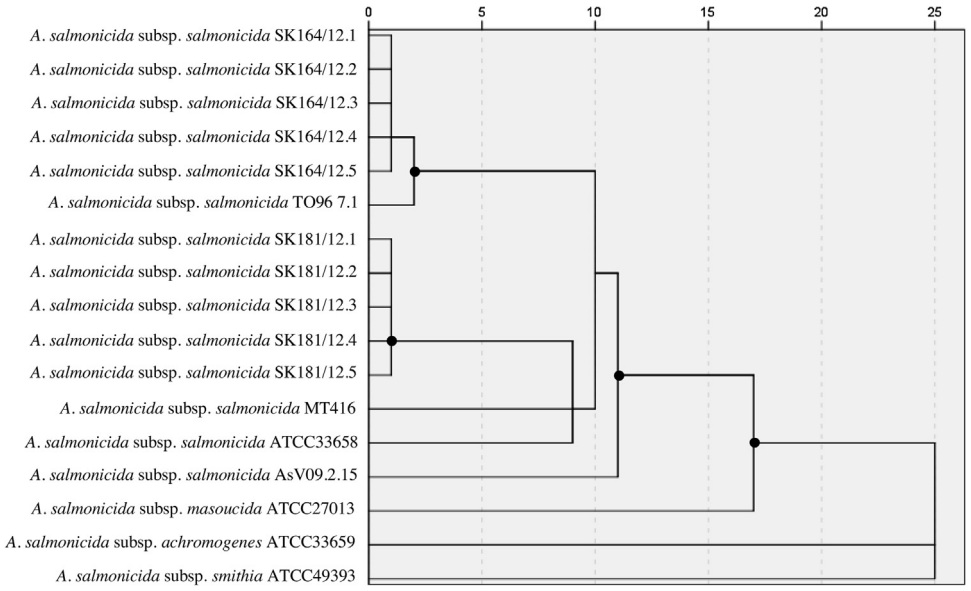

Fig. 2. Phyloproteomic tree of the subspecies of *A. salmonicida* used in this study using Pearson Product Moment Correlation Coefficient (PPMCC) and single linkage. The scale above the dendrogram indicates the relative distance used in the clustering analysis. Solid circles indicate that the corresponding nodes are also recovered using Jaccard similarity coefficient and single linkage.

regardless of their isolation source, grew on basal medium with lower concentrations of NaCl (0–3%), confirming their halotolerance (Delamare et al., 2000). However, sea bass isolates did not grow when higher concentrations of sodium chloride were used as already described for *A. salmonicida* subsp. *salmonicida* (Austin and Austin, 2007). The *A. salmonicida* subsp. *salmonicida* strains isolated from sea bass showed resistance to oxitetracycline. The appearance of resistance to oxytetracycline among *A. salmonicida* strains has been previously described (Adams et al., 1998; Austin and Austin, 2007).

Serological assays and analysis of cell envelope compounds (LPS and proteins) demonstrated that the isolated bacteria were antigenically similar to the reference strains of *A. salmonicida* subsp. *salmonicida* used for comparative purpose. Similar fatty acid profiles were obtained for all *A. salmonicida* subsp. *salmonicida* strains analysed, confirming the existence of high homogeneity within this subspecies.

At molecular level, *A. salmonicida* constitute a very uniform group, being very difficult to separate the subspecies on the basis of rRNA 16S, *gyrB* and *rpoB* sequence analysis (Benagli et al., 2012). In this study, the use of primers Fer-3 and Fer-4, which amplified a region of the *fstA* gene, allowed identifying all *A. salmonicida* strains tested but not its differentiation at subspecies level. Several authors (Benagli et al., 2012; Donohue et al., 2006) have reported that specific peak masses obtained from the MALDI-TOF MS analysis of intact-cell could be used as specific biomarkers for the

identification and differentiation among the species of *Aeromonas*. In the present study, MALDI-TOF MS analysis allowed the accurate identification of the bacterial strains isolated from sea bass as *A. salmonicida* subsp. *salmonicida* with score values ranging from 2.1 ± 0.07 to 2.39 ± 0.08. Interestingly, bacterial isolates from May 2012 showed a characteristic peak mass of m/z 2747.16, which was not detected in the other strains analysed. Furthermore, specific peak masses were identified that could allow discrimination of strains of *A. salmonicida* subsp. *salmonicida* from the reference strains of atypical subspecies analysed. Cluster analysis and corresponding dendrogram showed phyloproteomic relationships that reflect accurate classification of the strains analysed at subspecies level. These results suggest that MALDI-TOF analysis could be a reliable, low cost and less time consuming technique than conventional, serological and PCR-based methods for the diagnosis of typical furunculosis. Further studies, including a high number of strains, must be conducted to determine if the differences in proteomic profiles observed in this work are relevant for bacterial identification, epidemiology and/or pathogenesis. Moreover, the applicability of MALDI-TOF spectrometry in the direct detection of *A. salmonicida* in fish tissues, blood or mucus should be evaluated.

A. salmonicida subsp. *salmonicida* is the causative agent of furunculosis disease and affected salmonid fish commonly exhibit furuncle-like lesions (Austin and Austin, 2007). In our study, affected sea bass did not show the typical external sign of furunculosis in the first stages of the disease. However, when the disease progressed, open ulcers appeared on the skin and muscle as described in salmonid fish (Austin and Austin, 2007). It is commonly accepted that fish may contribute to disseminate the diseases by vertical and lateral transmission of the pathogens or by becoming carrier of *A. salmonicida* (Austin and Austin, 2007). Results of pathogenicity assays demonstrated that strains isolated from sea bass were virulent for sea bass, turbot and trout, when doses ranging from 2×10^4 to 2×10^7 CFU per fish were used. These results raise the question about the potential risks of transfer of the bacterium between fish species susceptible to furunculosis cultured in the same location as well as between farmed and wild fish.

It is noteworthy that, according to the information provided by the fish farmer, the use of an autogenous oil-adjuvanted vaccine is effective for the control of the furunculosis in sea bass, avoiding the appearance of new episodes in the affected farm.

Conflict of interest statement

The authors have not declared any conflict of interest.

Authorship

YS lead and supervised this study. MA collaborated in the isolation of the bacterium and the initial characterization of the pathogen. CF contributed substantially in the phenotypic, serological and molecular characterization of the isolated bacterium. The paper was initially written by CF and then was revised and corrected by YS and DG. All the authors read and approved the final version of the manuscript.

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