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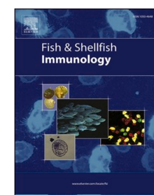

Full length article

Outer membrane vesicles (OMVs) from *Tenacibaculum maritimum* as a potential vaccine against fish tenacibaculosis

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A B S T R A C T

Outer membrane vesicles (OMVs) have been gained increasing attention in vaccinology due to their ability to induce strong protective humoral and cell-mediated immunity. The Gram-negative bacterium *Tenacibaculum maritimum*, the causative agent of marine tenacibaculosis, poses a significant challenge to the global aquaculture industry due to its difficult prophylaxis. In previous studies, we demonstrated that OMV production is a key virulence mechanism in *T. maritimum*. Building on this, the present study aimed to evaluate the efficacy of a natural, encapsulated multi-antigen vaccine made from adjuvant-free, crude *T. maritimum* OMVs (Tm-OMVs). A vaccination experiment using SP9.1-OMVs was conducted in juvenile turbot (*Scophthalmus maximus* L.), followed by a *T. maritimum* bath challenge. Immune responses in the turbot were assessed by measuring anti-Tm antibody levels and analyzing the expression of eight key immune-related genes (il-1β, il-8, il-22, pcna, c3, cd4-1, ifng2, cd8α). The results showed that immunization with SP9.1-OMVs provided significant protection against *T. maritimum* infection (RPS = 70 %). Vaccinated fish exhibited a dose-dependent increase in anti-Tm antibody titers in blood plasma, along with rapid induction of both innate (il-1β, il-8, il-22, c3) and adaptive (cd4-1, ifng2, cd8α) immune genes as early as 4 h post-bath challenge. These findings offer new insights into the early immune response of turbot following *T. maritimum* infection and could serve as a foundation for developing novel OMV-based vaccines.

1. Introduction

OMVs are nanosized spherical bilayer particles released by Gram-negative bacteria that contain biologically active components, such as lipopolysaccharide (LPS), phospholipids, and major outer membrane proteins (OMPs), as well as certain bacterial components that are entrapped during vesiculation [1–5]. OMVs have been receiving increasing attention in the field of vaccinology because they present bacterial antigens in native conformations and are able to induce potent protective humoral and cell-mediated immunity [6,7]. OMVs-based vaccines are being developed against animal and human pathogens including *Neisseria meningitidis*, *Klebsiella pneumoniae*, *Vibrio cholerae*, *Shigella sonnei,* or nontyphoidal *Salmonella* [8–12] and they are starting to be studied for fish vaccination [13–15].

Tenacibaculosis is a marine fish pathology caused by the Gram-negative bacterium *Tenacibaculum maritimum*. It is associated with skin lesions on the body of fish caused by long adherent filamentous bacterial mats that extend deeply into the connective tissue layer [16–18]. Due to the frequent outbreaks of tenacibaculosis worldwide, different approaches have been studied for its prevention and treatment. The aquaculture industry frequently uses autogenous vaccines formulated from formalin-inactivated bacterial cultures, despite the potential toxicity issues arising from the accumulation of toxins in the supernatants. Thus, the commercial bacterin-based vaccine ICTHIOVAC®-TM (HIPRA), derived from a serotype O2 strain, is being used although it gives only 6 months of immunity. Several autovaccines have been also tested [19]. However, all these vaccines have not demonstrated sufficient protective activity against *T. maritimum* [20–22].

T. maritimum has traditionally been considered a homogeneous bacterial species based on its morphological, physiological, and biochemical characteristics [16,23,24]. However, the existence of antigenic diversity in four serotypes (O1-O4) and eight sub-types

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complicates the development of effective vaccines for preventing tenacibaculosis [25–27]. Most of the strains causing epizootics belong to serotypes O1, O2, and O3, whereas serotype O4 was encountered only sporadically [24,25]. However, in recent years, strains of serotype O4 have been appearing more frequently [19]. For this reason, it has become necessary to evaluate the incidence of the different serotypes of this pathogen. In addition, recent results showed the existence of a large heterogeneity, particularly in the enzymatic capacity, within serotype O4 strains [28].

Adhesion to biotic and abiotic surfaces, gliding motility (T9SS), and production of extracellular products (ECPs) have been identified as contributors to *T. maritimum* virulence [18]. The synergistic action of toxins and enzymes present in ECPs is the main factor of *T. maritimum* pathogenesis, causing extensive damage to host tissues [24,29–31]. Characterization of the secretome of the serotype O4 *T. maritimum* SP9.1 revealed the production of outer membrane vesicles (OMVs) *in vitro*, which are specifically enriched in outer membrane proteins such as TonB-dependent transporters and components of the type IX secretion system (T9SS). In contrast, major proteo-/lipolytic functions, including sialidase SiaA, chondroitinase CslA, sphingomyelinase Sph, ceramidase Cer, and collagenase Col, were found exclusively in the soluble fraction of the ECPs [28]. These findings explain why the soluble fraction of the ECPs exhibits higher lytic activities.

To ensure broad protection against the diverse strains of *T. maritimum,* vaccine formulations need to include highly conserved antigens or a mixture of multiple proteins with the ability to stimulate the production of bactericidal antibodies effective against a wide range of circulating strains. As TBDTs are highly conserved as part of the outer membrane and OMVs in various bacterial species, they are attracting interest as vaccine antigens by functioning through the "siege" strategy, triggering an immune response against key features of the bacteria [32]. The aim of this study was to evaluate the efficacy of a *T. maritimum* OMV-based vaccine against tenacibaculosis using turbot (*Scophthalmus maximus* L.) as a model organism. The study confirmed the production of OMVs during *in vivo* infection and proved their role as natural encapsulated multi-antigen vaccine. Our findings shed light on new aspects of *T. maritimum* biology and may pave the way for the development of OMVs-based novel vaccines against tenacibaculosis and could be also of application against other bacterial diseases.

2. Materials and methods

2.1. Bacterial strains and culture Conditions

For this study, we used 200 *T. maritimum* strains isolated in our laboratory from 2000 to 2019 and some obtained from other laboratories (Table S1). Strain SP9.1 was isolated from diseased Atlantic salmon in Spain and selected as the experimental model. Collection strains NCIMB 2154^T (type strain) and NCIMB 2158 and strain PC503.1 (all from serotype O1), strain PC424.1 (serotype O2), strain ACC13.1 (serotype O3) and strain SNW20.2 (serotype O4) were used as control strains of each serotype. All strains were routinely cultured in FMM (*Flexibacter maritimus* Medium) agar or broth (Condalab) and incubated at 25 °C for 48 h. All strains were stored frozen in FMM broth with 8 % glycerol at −80 °C in single-use cryovials.

2.2. Serotyping analysis

The serological characterization of the *T. maritimum* isolates was performed using dot blot assay as described by Cipriano et al. [33] with slight modifications [34]. Briefly, the antigens were prepared from the resuspended strains (10^9 cells mL^{-1}) in 1 mL of saline solution (water and 0.85 % NaCl), subsequently incubated for 1 h at 100 °C and kept at 4 °C for subsequent use. Rabbit antisera raised against representative strains of the four serotypes described for this pathogen were previously obtained [19] according to the method described by Sørensen & Larsen [35] and used as control. One μL of the O-antigens obtained from each *T. maritimum* strain was dotted onto nitrocellulose membranes (0.45 μm HA filter, Millipore) previously washed with PBS (pH 7.4) and dried. Membranes were blocked for 1 h with 3 % gelatin (Oxoid) in Tris-buffered saline, TBS (pH 7.5). As negative control, 1 μL of PBS was used. The membranes were then washed twice with TBS-T and exposed to the anti-sera diluted 1:1000 in TBS with 1 % gelatin (TBS-1) for 60 min. The filters were then washed twice with TBS-T and incubated with goat anti-rabbit immunoglobulin G (diluted at 1:3000), with alkaline phosphatase as the conjugate (Bio-Rad). The immunoreactive point was visualized using 0.1 M carbonate buffer (pH 9.8) containing 4-nitro blue tetrazolium chloride (0.3 mg mL^{-1}, Oxoid) and 5-bromo-4-chloro-3-indolylphosphate (0.15 mg mL^{-1}, Oxoid). Once the appearance of the color was detected, the reaction was stopped by washing the membranes with distilled water. Only reactions of similar intensity to those obtained with control strains were considered positive.

2.3. Isolation of OMVs and protein quantification

T. maritimum OMVs (Tm-OMVs) were isolated as previously reported [28]. Briefly, cultures of 50 mL of *T. maritimum* strain SP9.1 were grown at 25 °C for 24 h in FMM (Condalab). These cultures were used to inoculate plates of FMM agar (23 × 23 cm), covered with a sterile cellophane sheet (Pacon) and incubated at 25 °C for 48 h. Bacteria of each plate were washed off the cellophane using 40 mL of PBS (pH 7.4), centrifuged at 4000 rpm for 30 min, and the pellet was discarded. Cell-free supernatants were centrifuged to collect the vesicles as a pellet by ultracentrifugation using a Beckmann SW32Ti rotor at 24,000 rpm at 4 °C for 2 h. OMVs (the pellet) were resuspended in 200 μL of PBS. Bacterial cultures and purification procedures were made in triplicate. OMVs samples were plated in FMM plates to verify that they were cell-free. The protein quantity in the original solution was measured using Qubit protein assay kit (Invitrogen, Paisley, UK) and diluted in sterile physiological saline solution (PBS) to reach a protein final concentration of 0.25 (Low OMVs concentration), 0.30 (Medium OMVs concentration) and 0.40 (High OMVs concentration) mg/mL.

2.4. Screening of TonB-dependent transporters in T. maritimum strains

Genes encoding 13 different TonB-dependent transporters [28] were detected by PCR in 64 *T. maritimum* strains (Table S2). Primers specific for each gene were designed using the Benchling software and are listed in Table S3. PCR reactions were performed using bacterial gDNA extracted using *InstaGeneTM* Matrix (Bio-Rad) according to the manufacturer's instructions. PCR reactions contained 12,5 μL NZYTaq II 2x Green Master Mix (NZYtech), 1 μL of each primer at 10 μM, 8,5 μL of miliQ H_2O and 2 μl of gDNA template in a 25 μL final reaction volume. The PCR amplification mix was heated at 95 °C for 3 min, followed by 35 cycles of 95 °C for 30 s, 58 °C for 30 s, 72 °C for 90 s, and a final extension at 72 °C for 5 min. The amplified products were electrophoresed in 2 % agarose gels run in 1X TBE buffer with the GeneRuler DNA ladder mix (Thermo Fisher Scientific) as a molecular size marker.

2.5. Vaccination assay

Turbot (*Scophthalmus maximus* L.) fingerlings (n = 200) with an average weight of 20 ± 2.3 g, and no previous history of tenacibaculosis, were purchased from a commercial hatchery and maintained in 200 L seawater tanks at a temperature of 20 ± 2 °C, salinity of 23–28 ‰, and a photoperiod of 14 h light:10 h dark. Fish were left to acclimate for 15 days prior to vaccination and their health status was monitored by observing external appearance, swimming behaviour, and appetite. The fish were fed on commercial pellets (Skretting), according to the supplier's recommendations. Water quality was maintained with mechanical and biological filtration and ozone-disinfection.

Four different groups (n = 50) of fish were used (Fig. 1) for

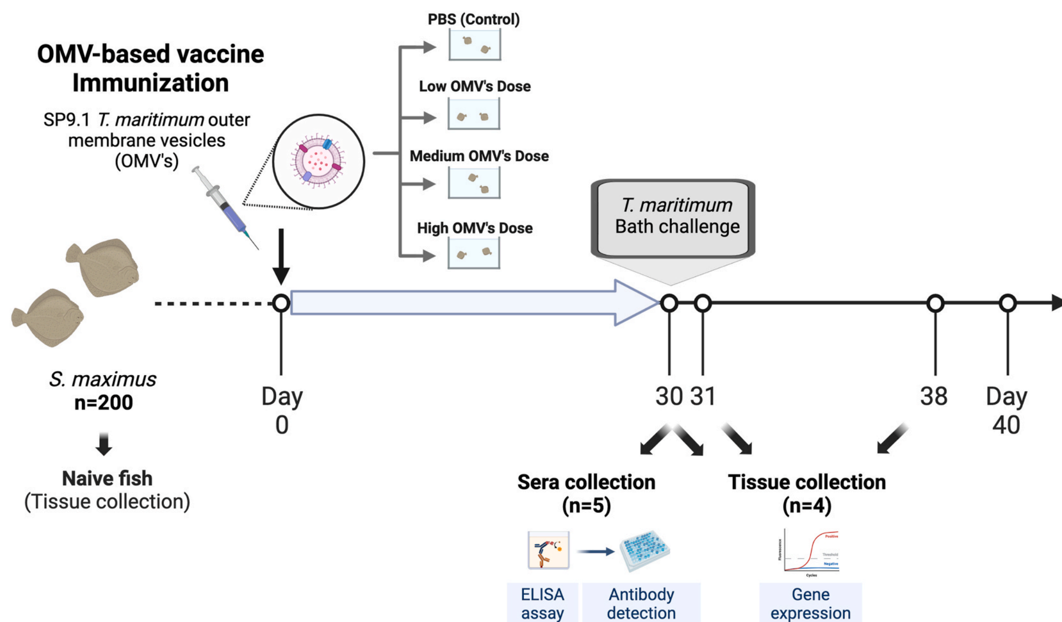


Fig. 1. Experimental procedure of turbot immunization with *T. maritimum* OMV's and subsequent *T. maritimum* challenge (1×10^6 CFU mL^{-1}). The timeline is given by a horizontal arrow from left to the right, starting with the initial immunization on day 0.

vaccination trials using different OMVs concentrations: Low OMVs dose (group 1), Medium OMVs dose (group 2) and High OMVs dose (group 3). A control group (4) was inoculated with PBS. In all cases a dose of 100 μL/fish were used. Intra-peritoneal injection (i.p.) of vaccines were performed under anesthesia achieved by immersion in 0.03 % (v/v) 2-fenoxyethanol. Euthanasia was performed by immersion in 0.06 % (v/v) 2-fenoxyethanol, followed by bleeding. The possible cytotoxic effect of the solutions was controlled by monitoring the fish health status in the hours after immunization.

All experimental protocols involving fish were carried out in accordance with European and Spanish legislation for the use of animals for scientific purposes (Directive 2010/63/EU) and were approved by the Bioethics Committee of the University of Santiago de Compostela (Protocol Nº 15012/2022/001).

2.6. LD_{50} determination

For determining the lethal dose 50 of *T. maritimum* infections, three different doses (1×10^5, 1×10^6 and 1×10^7 CFU mL^{-1}) from exponential-phase cultures of SP9.1 strain were tested in some individuals (*S. maximus*; mean weight 20 g). Groups of 10 fish were bath challenged in 1 L of sea water at 20 ± 2 °C with strong aeration for 2 h. Afterwards, the rearing water in each tank was discarded and fish were placed in new tanks with clean water. The recirculation system was re-established. The survival rate was observed every 12 h for 7 days. Skin fish with physical symptoms of tenacibaculosis (ulcers) were collected to check the presence of OMVs by SEM. This assay was repeated twice to verify the results. LD_{50} was calculated according to the method of Reed & Muench [36]. The selected dose for the experimental infections was 1×10^6 CFU mL^{-1}.

2.7. Relative survival percentage assays

One month after vaccination, fish from all groups (control, Low OMVs dose, Medium OMVs dose and High OMVs dose) were bath challenged with a final bacterial concentration of 1×10^6 CFU mL^{-1} to assess vaccine efficacy and maintained in 200 L seawater tanks at a temperature of 20 ± 2 °C, salinity of 23–28 ‰, and a photoperiod of 14 h light:10 h dark with strong aeration. Fish were fed daily at a ratio of 1 % of total fish biomass. Water was changed each day to prevent bacterial accumulation. In addition, ammonia and nitrite levels were assessed daily and kept below 0.025 and 0.3 mg L^{-1}, respectively. Fish were monitored for 10 days at least three times a day, and mortalities were recorded. The specificity of mortalities was confirmed by a PCR targeting the *tly* gene from internal organs (kidney) of dead or moribund fish. The PCR protocol used for amplification and the primers used are detailed in Tables S4 and S5. Vaccine efficacy was evaluated by determining the relative percent survival (RPS), as follows: RPS = [1 − (% mortality in vaccinated group/% mortality in control group)] × 100. The cumulative probabilities of survival were analyzed using the Kaplan–Meier survival curves and group comparisons were performed using the log-rank test. Student's t-test was used to calculate p-values based on comparisons with the control. Differences between groups were considered statistically significant when $p < 0.05$.

2.8. Assessment of anti-Tm antibody levels

Thirty days after the immunization, blood samples were collected from the caudal vein of 5 fish per group (control, Low, Medium, and High dose) with 1 mL syringes and placed in 1.5 mL, allowed to clot for 24 h at 4 °C, and centrifuged at 5000×*g*, 5 min, 4 °C (Fig. 1). Sera were collected and stored at −80 °C. The content of anti-*Tm* igM in sera was determined by ELISA. Briefly, 96-well micro-titer plates were coated with 50 μL of *T. maritimum* cells representative of the 4 serotypes (O1, O2, O3 and O4), suspended in FMM at an OD_{600} of 0.8, corresponding to approximately 10^8 bacterial cells mL^{-1}. Two different *T. maritimum* strains were selected for each serotype (Table 1). Cells of each strain

Table 1
T. maritimum strains used in the present study.

STRAIN	HOST	SEROTYPE	ORIGIN	YEAR
NCIMB 2154^T	*Pagrus major*	O1	Japan	1977
LM4.1	*Scophthalmus maximus*	O1	Spain	2007
ACR570.1	*Solea senegalensis*	O2	Spain	2014
PC424.1	*Scophthalmus maximus*	O2	Spain	2019
ACC13	*Solea senegalensis*	O3	Portugal	2004
SNW5.1	*Salmo salar*	O3	Spain	2014
SP9.1	*Salmo salar*	O4	Spain	1993
VIL3.1	*Scophthalmus maximus*	O4	Spain	2004

were formalized prior to ELISA assay by addition of 1 % (v/v) formalin (Panreac) to each *T. maritimum* culture, followed by adjustment to an OD_{600} value of 1.0 using PBS and then used as antigens for antibody quantification. Wells were blocked with 200 μL of 2 % BSA in PBS containing 0.1 % Tween 20 (PBS-T) and were incubated for 2 h at RT with 50 μL of the serum to be tested diluted 1:100 in antibody solution (1 % BSA in PBS-T). Turbot immunoglobulins were detected with the mouse anti-turbot IgM monoclonal antibody (1:100 in antibody solution, Aquatic Diagnostics Ltd ref #2106/F08/03), followed by a horseradish peroxidase-conjugated rabbit anti-mouse polyclonal IgG (Dako ref #P0260; 1:2000 in antibody solution). Finally, 50 μL of Ultrasensitive TMB solution (ES022, Merck) was added to each well. The reaction was stopped by adding 50 μL of 0.3 M H_2SO_4 and A_{450} measured in an iMark™ Microplate Absorbance Reader (BioRad). Comparisons between antibody levels in sera from vaccinated and control groups were performed using the Mann–Whitney test and differences were considered significant when $p < 0.05$.

2.9. Quantitative real-time PCR

Real-time quantitative PCR (qRT-PCR) was carried out to visualize the relative gene expression levels of immune related genes in the fish after the challenge. Four fish (biological replicates) were removed from the high-OMVs-dose at 5-, 24 h and 8 days post-challenge to collect tissues (kidneys) for RT-qPCR analysis. Head kidneys were also collected before immunization and used as controls (naïve fish). Samples were preserved in 'RNA-later' solution (Ambion, Austin, TX) at 4 °C for the first 24 h, and then stored at −80 °C for molecular biology analysis. Total RNA was isolated from kidney samples using the TRIzol reagent (Invitrogen, Scotland, UK) according to the manufacturer's protocol. Quality and quantity of the RNA were analyzed by agarose gel electrophoresis (1.5 %), spectrophotometric analysis at 260 nm and 280 nm to ensure the 260:280 nm ratio and measured using Qubit protein assay kit (Invitrogen, Paisley, UK). Primers to amplify genes related with adaptive and innate immune response in turbot were selected from previous works and are listed in Table 2 [37–39]. RT-qPCR was achieved in duplicates (technical replicates) using One-step NZYSpeedy RT-qPCR Green Kit (NZYtech), according to the manufacturers' instructions. Briefly, qRT-PCRs were performed using 5 μL of diluted RNA mixed with 10 μL of One-Step NZYSpeedy qPCR Green master mix (2x), 0.8 μL of NZYRT mix, 7.6 μL of Nuclease-free water and 0.8 μL (10 μM) of each primer in a final volume of 25 μL. The amplification steps started with a reverse transcription at 50 °C for 20 min, a polymerase activation at 95 °C for 5 min, 40 cycles of denaturation at 95 °C for 5 s followed by annealing at 58 °C for 50 s. Melting curve analysis was performed to identify the specificity of PCR products and primers efficiency was calculated. Using Ubiquitin (*ub*) as an internal reference gene, the relative expression of each gene in kidney tissues was determined by the $2^{-\Delta\Delta Ct}$ calculation technique based on the Ct values obtained by RT-qPCR [40]. The Analysis of Variance (ANOVA) test was used to assess the significance of differences between the treatment and control groups by Statistical Product and Service Solutions (SPSS) 19.0 (SPSS, Chicago, IL, USA). Differences were considered significant when $p < 0.05$ and $FC > 1.50$.

2.10. Scanning electron microscopy (SEM)

The presence of OMVs of *T. maritimum* strain SP9.1 in skin during the infection was examined by SEM. SEM sample preparation was performed according to a previously described method with some modifications [41]. Fish tails were collected after bath challenge at 2, 7 and 24 h, and stored at −80 °C. Tail samples from a non-infected fish were also collected as controls. Tissues attached to coverslip surfaces were fixed in 2 % (v/v) glutaraldehyde in 0.1 M of cacodylate buffer (pH 7.4). Then, samples were post-fixed with 1 % osmium tetroxide for 2 h. Finally, they were dehydrated with a series of graded ethanol solutions, starting with 50 % and followed by 70 %, 75 %, 96 %, and 100 % (× 3), and they were air-dried after a final overnight dehydration. Samples were sputter-coated with iron and then examined by SEM (FESEM Ultraplus, ZEISS, Jena, Germany) at an acceleration voltage of 3 kV and different magnifications (x10,000; x30,000; x50,000). All microscopic examinations were done at the Electron Microscopy facilities (RIAIDT) of the University of Santiago de Compostela.

3. Results

3.1. Serotyping analysis reveals an increasing incidence of serotype O4 strains

To conduct an epidemiological analysis of the *T. maritimum* strains isolated from tenacibaculosis outbreaks, the serotype of 200 strains isolated during the period between 2000 and 2019 by the Aquatic One Health Research Center (iARCUS-USC) was determined using dot-blot. The strains were isolated from diverse origins and host species (Table S1). The number of strains by serotype and their time distribution is depicted in Fig. 2. All strains tested showed a clear and strong reaction of the O antigens with their homologous serum. Most of the strains isolated between 2000 and 2019 belong to serotype O2 (46.5 %), followed by serotypes O3 (20.7 %), O1 (20.7 %), and O4 (12.1 %) (Fig. 2A). It is noteworthy that strains of serotypes O2 and O3 were consistently isolated throughout the entire study period (2000–2019). Interestingly, strains belonging to serotype O1 were primarily isolated during the

Table 2
Oligonucleotide sequences used in the present study to assess immune response in turbot kidneys.

Protein	Gene	Accession Number	Cell	Sequence (5'→3')
Ubiquitin	*ub*	XP_035493997.2	All	F: GCGTGGTGGCATCATTGAGC R: CTTCTTCTTGCGGCAGTTGACAG
Interleukin 1 Beta	*il-1β*	AJ295836	Monocytes and macrophages	F: TACCTGTCGTGCCAACAGGAA R: TGATGTACCAGTTGGGGAA
Interleukin 8	*il-8*	XM_035651350.1	Various cell types	F: GGAGCCCAGAGTGTCGTTA R: CCTGCACCATAGAAATCTCATT
Proliferating Cell Nuclear Antigen	*pcna*	EU711051.1	Hematopoietic progenitor and other cells	F: GGTGGACGAGTGCTGCTTC R: CTGCCCTGCGGTACTAACC
Complement Factor 3	*c3*	XM_035636545.1	Complement component	F: GGTACAACTTCAACAACAACAACAA R: AGCGTAGTACAGCGACACCATT
Type II interferon gamma	*ifng2*	XM_035604040.2	T-cells and NK cells	F: GACACCTCGGGAGTTCTGTG R: CTGAATCATCCGAGGGGACG
Proinflammatory cytokine	*il-22*	XM_035612680.1	T helper cells	F: TTGTTTTGAACTCCTCTGTGTGT R: GGATGGTGTCACCGTGGAAA
Immunoglobulin CD4	*cd4*	XM_035642165.2	T cells	F: C ACAGACAATCACTTGTGACTACGA R: CGACCGTATACCACCTCAGC
T lymphocyte glycoprotein CD8	*cd8α*	XM_035630766.2	T cells	F: AACGCTCTGCTGCAATGTTC R: G TCCCCCTCTTTCACGACTCT

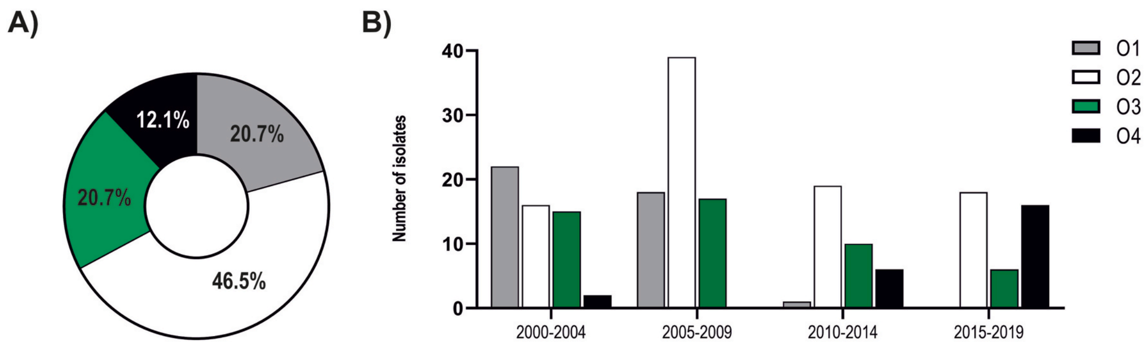


Fig. 2. Epidemiological study in a collection of 200 *Tenacibaculum maritimum* isolates. (A) Strains isolated between 2000 and 2019 grouped by serotypes (O1-O4) and (B) serotype occurrence grouped by 4-year periods. The specific data of the strains are reported in Table S1.

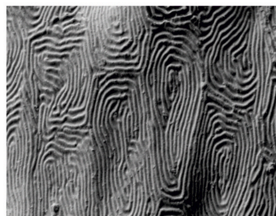
initial years (2000–2009), with a decrease observed in the more recent periods analyzed (2010–2019). Conversely, serotype O4 strains were only sporadically isolated in 2000–2009, but the number of isolates increased significantly from 2010 onwards, making it one of the most represented serotypes in the period 2015–2019 (Fig. 2B). Our results also showed that isolates of *T. maritimum* belonging to different serotypes coexist in the same host and in the same fish farm (Table S1). Thus, vaccines developed against a single serotype may not be effective enough against this pathogen.

3.2. T. maritimum produces OMVs in fish tissues during infection

T. maritimum SP9.1 strain, belonging to serotype O4, was previously used to assess *in vitro* OMVs production by SEM and TEM and to characterize the complete secretome of this species [28]. In this work, the *in vivo* production of OMVs in fish tissues was confirmed during an experimental infection using the same strain (Fig. 3). Skin samples from turbot were collected at various time points after bath inoculation and analyzed using scanning electron microscopy (SEM). Two hours post-bath, individual *T. maritimum* cells adhering to the fish skin were found (Fig. 3b). More notably, surface blebbing, indicative of outer membrane vesicle production in *in vitro* assays, were observed at 2- and

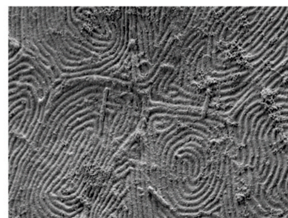
A) Before challenge

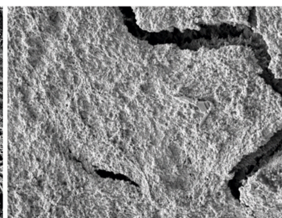
Control

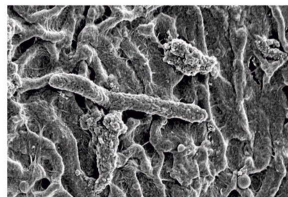


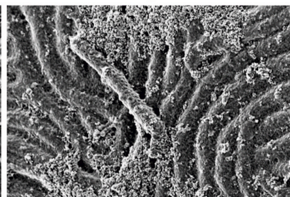
B) Post-challenge (h)

2 7 24









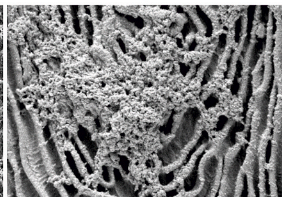


Fig. 3. Observation by SEM of turbot skin infected with *T. maritimum*. (a) SEM image from a turbot tail without tenacibaculosis symptoms. (b) Turbot tail sample after infection with *T. maritimum* by bath at 2, 7 and 24 h post-infection.

7-h post-bath. Over time, during colonization, the skin surface becomes covered by vesicles that adhere to each other, forming a dense matrix or biofilm at 24-h post-bath. These images resemble *T. maritimum* surface colonization previously observed *in vitro* [28].

3.3. OMVs from serotype O4 strain SP9.1 induce high antibody levels against all T. maritimum serotypes

Previous studies performed in our group reported that SP9-OMVs are enriched in 23 TonB-dependent transporters (TBDTs) [28]. These type of outer membrane proteins are typically immunogenic [32]. To study if these genes are conserved in *T. maritimum* species, PCRs of 13 of these genes, encompassing to the most abundant TBDT in the OMV's, were tested in 64 *T. maritimum* strains (Table S2). PCR results showed the widespread presence of these TBDT genes in almost all *T. maritimum* strains assayed, regardless of the serotype, host species or place of isolation (Table S2).

To test the use of *T. maritimun* SP9.1 OMVs -charged with these TBDT- as a possible vaccine formulation, three groups of 50 turbot fingerlings were immunized by IP with different doses of SP9.1 OMVs per fish: 25 μg (Low dose, L), 30 μg (Medium dose, M), or 40 μg (High dose, H). A non-immunized control group was treated with PBS (Control, C). The experimental design used to evaluate immune response and protection is illustrated in Fig. 1. Unfortunately, the IP administration of a bacterin formulated with inactivated cultures of *T. maritimun* SP9.1, or with OMV preparations at concentrations ≥45 μg per fish, showed toxicity in turbot (data not shown). Thirty days after immunization, the antibody level in sera of fish treated with OMVs were significantly higher against all serotypes than those found in the non-immunized control group (Fig. 4). Additionally, the immune response exhibited a dose-response relationship with the OMV dose, with the highest antibody level observed in the fish group immunized with the higher OMV dose (40 μg/mL). As expected, the maximal antibody level was observed against whole cells of strain SP9.1, with an A_{450} of ca. 2.5 at the lower dose. Notably, immunization with the SP9.1 OMVs at medium or high doses also induced significantly high antibody levels against all the *T. maritimum* strains tested (Fig. 4), regardless of their serotype, showing antibody levels of $A_{450} \geq 1.5$.

3.4. SP9.1 OMV-based vaccine confers protection against tenacibaculosis

To evaluate the protective efficacy of the SP9.1 OMV-based vaccine against tenacibaculosis, an infection challenge using a LD_{50} of the pathogen was conducted and fish health status was monitored twice daily for 10 days post-infection. Fish of the PBS control group exhibited more pronounced signals of tenacibaculosis (morbidity) (Fig. 5A) and the highest mortality rates (Fig. 5B). The fish of this control group began to die 1-day post-infection and the cumulative survival achieved 40 % on day 5-post challenge (Fig. 5B). The fish group immunized with the lower dose of OMVs showed a survival curve statistically indistinguishable from the non-immunized control ($p = 0.1359$), giving a cumulative survival of 46 % on day 6 (Fig. 4). However, significant differences in the survival curves were detected between the non-immunized control and those fish groups immunized with the medium or the high doses of the SP9.1 OMV-based vaccine ($p = 0.0390$), which

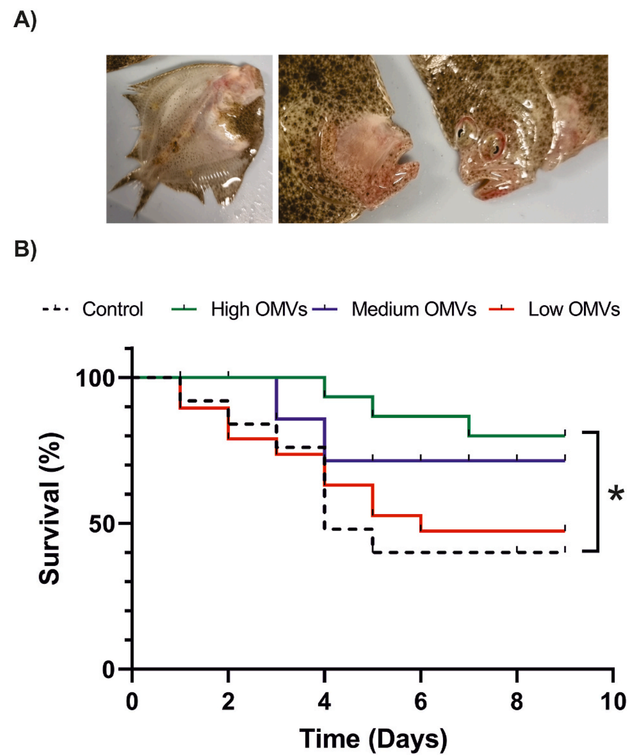


Fig. 5. (A) Representative images of turbot (*Scophthalmus maximus*) showing clinical signs of tenacibaculosis after the immersion challenge. Haemorrhages can be observed predominately around the jaws, and on the flanks and fins. (B) Kaplan-Maier survival analysis (accumulative survival) after immersion challenge of turbot groups with an LD_{50} of *T. maritimum* SP9.1. Lines represent the accumulative survival rates of fish groups immunized with low (L), medium (M) or high (H) dose of SP9.1 OMV-based vaccine, or with PBS (Control). The existence of statistically significant differences was detected using Kaplan-Maier test and denoted with an asterisk (*, $p < 0.05$).

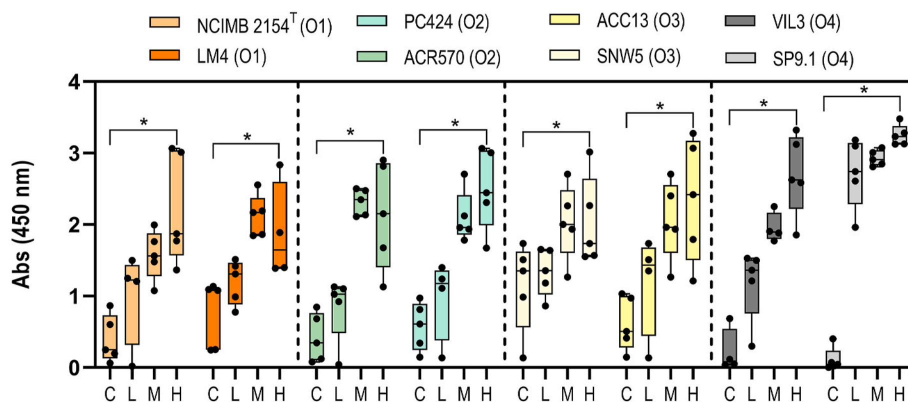


Fig. 4. Antibody levels measured by ELISA (A_{450}) in sera of fish immunized with SP9.1 OMV-based vaccine at low (L), medium (M), or high (H) dose, as well as the control group treated with PBS (C), against whole cells of *T. maritimum* strains representative of the main serotypes (Table 1). An asterisk (*) indicates the presence of statistically significant differences compared to the unvaccinated control, as determined by the *t*-student test (*P* value < 0.05). Vertical bars indicate the median of each data set.

exhibited a survival percentage of 78 % and 82 %, corresponding to a RPS of 63 % and 70 %, respectively (Fig. 5B). The typical symptoms of tenacibaculosis, including gnawed tails and red mucus [42], were primarily observed in the fish of the non-vaccinated control and in those vaccinated with the low dose of OMVs (Fig. 5B). Skin lesions exhibited long filamentous structures forming adherent bacterial mats over the eroded surface, and *T. maritimum* was recovered from them. Finally, the pathogen was detected in the internal organs (kidneys) of the dead fish using a PCR targeting tenacilysin gene. In contrast, almost no clinical symptoms were observed in the fish of the groups vaccinated with the medium or high dose of the SP9.1 OMV-based vaccine.

3.5. Fish immunized with SP9.1 OMV-based vaccine exhibit a quicker activation of innate and adaptive immune responses upon pathogen challenge

To evaluate the activation of the innate and adaptive immune response during an infection challenge in fish vaccinated with OMVs, we analyzed a basic profile of expression of immune-related genes (Table 2). For this purpose, kidney samples from 4 fish in both, the non-immunized control (treated with PBS) and high dose of OMVs, were individually harvested at 5-, 24-, and 192-h (8 days) post-challenge. The expression of these immune-related genes involved in inflammation, cellular activation or differentiation were evaluated and compared to naïve fish, which were neither immunized nor challenged (Fig. 1). The results reveal early upregulation of genes such as *pcna* (proliferating cell nuclear antigen), inf*2* (interferon gamma), *c3* (major activator complement component C3), *cd4* and *cd8* (clusters of differentiation CD4 and CD8, respectively) in the fish treated with the SP9.1 OMV-based vaccine, beginning from 5 h post-pathogen exposure and peaking at 24 h post-challenge (Fig. 6). On the other hand, these genes on the naïve fish started up-regulation significantly later, at 8 days post-bath challenge. Proinflammatory cytokine *(il-22)* presented upregulation at 24 h and peaked at 8 days from challenge in vaccinated fish. Interleukin 8 (*il8*) was not significantly induced in any group. It was noteworthy the elevated expression levels of the pro-inflammatory cytokine *il-1β* and the cluster of differentiation 8 (*cd8*) in the fish group treated with the SP9.1 OMV-based vaccine, showing 28- and 33-fold higher expression values, respectively, at 24 h post-challenge (Fig. 6). Furthermore, the central complement activator component, *c3*, achieved its maximal expression value at this time point, with an 11.1-fold increase in expression. In contrast, in the PBS-treated control the genes *il-1β*, *c3* and *cd8* barely reached overexpression values of 2.8, 4.9, and 12.5, respectively, at 8 days post-challenge. Among the most activated genes in PBS-treated control were also found *pcna* and inf*2*, achieving 8.3- and 7.7-fold increase after 8 days. These results altogether greatly suggest a faster and robust activation of both innate and adaptive immune systems following pathogen exposure in the fish treated with the SP9.1 OMV-based vaccine, compared to non-vaccinated fish.

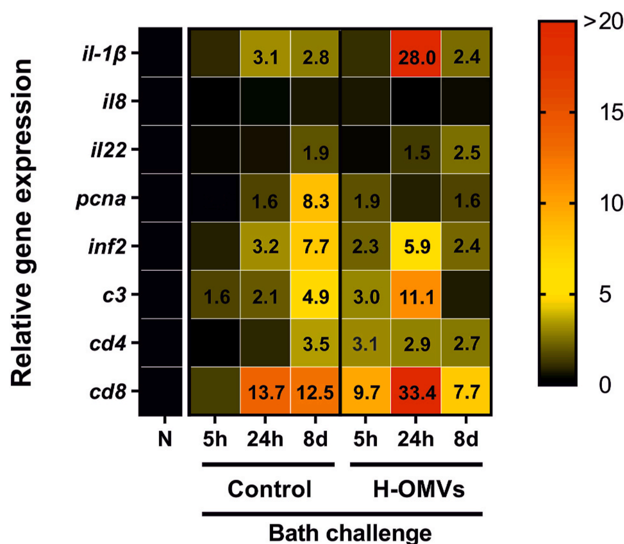


Fig. 6. Differential gene expression profile in fish control group (Control), treated with PBS, and high dose of SP9.1 OMV-based vaccine (H-OMVs) at various time points post-challenge (5 h; 24 h; or 8 days, 8d) compared to naïve fish (N), which did not receive any treatment. Expression values of transcripts involved in inflammation, cellular activation and differentiation were evaluated in head kidney tissues and showed in two colour maps. Expression values were normalized to *ub* mRNA expression. Fold-change values significantly different from the naïve fish are presented as the mean of four independent replicates (A double criteria of fold change cut-off $\geq$1.5 and *t*-test, $p < 0.05$).

4. Discussion

The most used vaccines against *T. maritimum* are bacterins, most of which are autogenous, composed of formalin-inactivated cells from O1 and O2 serotypes. However, they have shown limited success [43–45]. The ICTHIOVAC®-TM (HIPRA) vaccine, designed to prevent mortality in turbot, demonstrated the most satisfactory results. It provides a relative percent survival (RPS) of 75 % and a duration of immunity of 6 months [19]. However, the antigenic diversity of *T. maritimum* significantly complicates vaccine development and efficacy against different serotypes [27]. Our serotyping analysis (Table S1) aligns with the serotype distribution previously reported by Avendaño-Herrera et al. [25] and Castro et al. [26], confirming that strains belonging to serotype O2 are consistently the most isolated from tenacibaculosis outbreaks. More interestingly, our findings show a growing occurrence of epizootics associated with strains of serotype O4 and the coexistence of *T. maritimum* strains of various serotypes within the same fish farms (Table S1 and Fig. 2). The emergence of serotype O4 may potentially be linked to the ineffectiveness of existing vaccines and autogenous vaccines against tenacibaculosis in aquaculture facilities.

Despite the significant potential of OMVs for vaccine development, there are only a few studies on OMV-based vaccines for fish. Park et al [14] showed that the administration of OMVs from the fish pathogen *Edwardsiella tarda* to olive flounder (*Paralichthys olivaceus*) conferred protection against experimental edwardsiellosis (RPS of 70 % and 70 % of fish survived). Brudal et al. [13] tested the use of OMVs as vaccines against francisellosis using zebrafish as model. More recently, vaccination of sea bass (*Dicentrarchus labrax*) with adjuvant-free crude OMVs showed the production of anti-*Phdp* antibodies against *Phdp* infection but reaching lower protection RPS values (38.2 %) [15]. We previously showed that *T. maritimum* produces high amounts of OMVs *in vitro*, which likely play a key role in virulence by facilitating surface adhesion and biofilm formation [28]. The present work reinforces this hypothesis, since a high production of OMVs was observed during the early stages of fish colonization (Fig. 3). Notably, we demonstrated that the SP9.1 OMV-based vaccine, particularly at medium (0.30 mg/mL) and high (0.40 mg/mL) doses, can stimulate both innate and adaptive immune responses, leading to elevated antibody levels against all *T. maritimum* serotypes (Fig. 4). We hypothesize that this strong immunogenicity may be due to the abundance of TBDTs present in *T. maritimum* OMVs. TBDTs act as virulence factors primarily responsible for the uptake of iron–siderophore complexes, heme, and vitamin B12 [46], and are often regarded as excellent candidates for vaccine development against Gram-negative bacteria [32]. The genome of *T. maritimum* SP9.1 strain encodes 23 distinct TBDTs, which are major components of OMVs [28]. Interestingly, they were also found to be widespread in a collection of *T. maritimum* strains, regardless of their geographical origin, host, or serotype (Table S1). BLAST searches of the NCBI database indicate that these proteins are highly conserved among *T. maritimum* isolates, showing amino acid identities greater than 98 % (data not shown). These results suggest that the SP9.1 OMV-based vaccine could be effective against a wide range of *T. maritimum* strains. However, further

studies are necessary to determine antibody specificity, assess the duration of protective immunity, and evaluate its efficacy against heterologous strains. Otherwise, several virulence factors, including proteolytic and lipolytic functions, were also found in association with the OMVs [28]. This suggests that these OMVs may have the potential to inhibit host immune responses or cause toxic effects. In fact, the SP9.1-based vaccine was found to be toxic at concentrations above 0.45 mg/mL. Brudal et al. [13] also reported toxic effects and proposed that further purification of the vesicles could reduce toxicity. However, such a strategy would increase the cost of OMV production, making it less feasible for use in aquaculture. One advantage of OMVs is that they can be engineered to remove irrelevant or harmful antigens and toxic components, or to incorporate autologous or heterologous protective antigens, further expanding their potential for vaccine development [47].

While the mechanisms underlying the long-term protection elicited by vaccines in fish remain poorly understood, the efficient stimulation of both the humoral and adaptive immune systems typically provides long-lasting immunity [48]. The fish group immunized with the high dose of SP9.1-OMVs-based vaccine not only exhibited significantly high antibody titters against the whole cells of *T. maritimum* (Fig. 4), but also an early upregulation of immune-related genes following exposure to the pathogen (Fig. 6). We included the analysis of proinflammatory cytokine (IL-22), Interleukins (IL-1β and Il-8), proliferating cell nuclear antigen (PCNA), interferon gamma (INF2), major activator complement component C3, and clusters of differentiation CD4 and CD8, which globally mark the innate and adaptative immune response [38,39]. Our findings collectively greatly suggest that the SP9.1 OMV-based vaccine induces a robust state of innate and adaptive immune activation, effectively promoting both cellular and humoral immune responses within the early hours following pathogen exposure and are consistent with immune responses described in other studies following immunization with *T. maritimum* bacterins. Faílde et al. [49] revealed that subcutaneous infection of turbot with *T. maritimum* provoked a humoral immune response that resulted in an increase in leukocytes in peripheral blood and in the number and distribution of Ig + cells in the spleen, kidney, thymus and intestine. Guardiola et al. [50] carried out a virulence test by bathing that showed a delay in the immune response of the sole mucus, and a significant increase in neutrophils after 48 and 72 h. Subsequently, Escribano et al. [51] revealed that sole bath immersion performed with *T. maritimum* influenced some innate immune parameters in skin mucus of bath challenged fish compared to unchallenged ones. On the other hand, Ferreira et al. [52] suggested that *T. maritimum* induces a local innate immune response (*il-1β, il8, mmp9* and *hamp1*) upon bath infection not only in the skin of European sea bass, but also in the gills and posterior-intestine, likely triggered by the *T. maritimum* capacity to adhere, colonize and damage these organs that can function as entry ways to bacteria, leading ultimately to the seen host's systemic response. The faster activation of both humoral and cellular immune responses in vaccinated fish after exposure to the pathogen correlates with significant protection against tenacibaculosis, ultimately resulting in a relative survival rate (RPS) of 70 % and an approximately survival percentage of 82 % during experimental infection challenge (Fig. 5B). Hence, immunization with the SP9.1 OMV-based vaccine could effectively reduce both morbidity and mortality caused by *T. maritimum* (Fig. 5). The obtained results align with the evaluation of fish immune response, as infection challenge outcomes correspond with the induction of immune response, particularly with antibody titers (Figs. 4 and 6). Therefore, we can conclude that the utilization of OMV-based vaccines could be instrumental in decreasing the incidence of infections caused by *T. maritimum*.

5. Conclusions

OMVs can act as natural, encapsulated multi-antigen vaccines, as they are enriched with cell surface proteins, including virulence-related factors like outer membrane TonB-dependent transporters (TBDTs). It has been demonstrated that the Tm-OMVs vaccine can generate significantly elevated antibody titers against whole cells from *T. maritimum* strains across all characterized serotypes (O1-O4), particularly at medium and high doses. Our findings strongly suggest that utilization of *T. maritimum* OMV-based vaccines could offer a broader-spectrum prophylactic approach to reduce the incidence of tenacibaculosis, regardless of the strain serotype. The immunogenicity of OMV-based vaccines is attributed to the high concentration of pathogen-associated molecular patterns (PAMPs) in the vesicles, along with their intrinsic adjuvant properties, which avoid the adverse effects commonly associated with conventional immune-enhancing adjuvants. These promising results highlight the potential of OMV-based vaccines in aquaculture.

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CRediT authorship contribution statement

M. Pilar Escribano: Data curation, Formal analysis, Investigation, Methodology, Validation, Writing – original draft, Writing – review & editing. **Miguel Balado:** Conceptualization, Formal analysis, Investigation, Methodology, Validation, Writing – original draft, Writing – review & editing. **Beatriz Santos:** Data curation, Investigation, Methodology. **Alicia E. Toranzo:** Funding acquisition, Project administration, Supervision. **Manuel L. Lemos:** Conceptualization, Funding acquisition, Project administration, Supervision, Validation, Writing – review & editing. **Beatriz Magariños:** Conceptualization, Funding acquisition, Project administration, Supervision, Validation, Writing – review & editing, All authors contributed to the article and approved the submitted version.

Declaration of competing interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2024.109943.

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