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Vibrogen-2 vaccine trial in lumpfish (*Cyclopterus lumpus*) against *Vibrio anguillarum*

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Abstract

Lumpfish (*Cyclopterus lumpus*), a native fish of the North Atlantic Ocean, is utilized as cleaner fish to biocontrol sea lice infestations in Atlantic salmon aquaculture. However, bacterial infections are affecting cleaner fish performance. *Vibrio anguillarum*, the aetiological agent of vibriosis, is one of the most frequent bacterial infections in lumpfish, and effective vaccine programmes against this pathogen have been identified as a high priority for lumpfish. Vibrogen-2 is a commercial polyvalent bath vaccine that contains formalin-inactivated cultures of *V. anguillarum* serotypes O1 and O2, and *Vibrio ordalii*. In this study, we evaluated Vibrogen-2 efficacy in lumpfish against a local isolated *V. anguillarum* strain. Two groups of 125 lumpfish were bath-immunized, bath-boost-immunized at four weeks post-primary immunization, and intraperitoneally (i.p.) boost-immunized at eight weeks post-primary immunization. The control groups were i.p. mock-immunized with PBS. Twenty-seven weeks post-primary immunization, the fish were i.p. challenged with 10 or 100 times the *V. anguillarum* J360 LD_{50} dose. After the challenge, survival was monitored daily, and samples of tissues were collected at ten days post-challenge. Commercial vaccine Vibrogen-2 reduced *V. anguillarum* tissue colonization and delayed mortality but did not confer immune protection to *C. lumpus* against the *V. anguillarum* i.p. challenge.

KEYWORDS
cleaner fish, lumpfish, vaccine, *Vibrio anguillarum*, Vibrogen-2

1 | INTRODUCTION

Even though globally aquaculture is the fastest-growing food-production industry (FAO, 2018), parasitic diseases, like sea lice infestation (*Lepeophtheirus salmonis*, *Caligus elongatus*, etc.), plague this sector (Fisheries & Oceans Canada, 2017; Stentiford et al., 2017; Wootten, Smith, & Needham, 2011). Sea–lice is the greatest health challenge limiting production of the Canadian Atlantic salmon (*Salmo salar*) aquaculture industry since the 1970s (Brandal & Egidius, 1977; Fisheries & Oceans Canada, 2013; Mustafa, Rankaduwa, & Campbell, 2001; Nilsen, Nielsen, Biering, & Bergheim, 2017; Powell et al., 2018; Torrissen et al., 2013). Sea–lice is a copepod ectoparasite (Aaen, Helgesen, Bakke, Kaur, & Horsberg, 2015; Hamre et al., 2013; Nilsen et al., 2017; Wootten, Smith, & Needham, 2011) that immune compromises the fish host, increasing susceptibility to viral and bacterial infections (Brooker et al., 2018) and causing significant losses and high treatment costs (Costello, 2009; Fisheries & Oceans Canada, 2017). It has been estimated that sea–lice infection results in up to a 16% reduction in production biomass, which is approximately equivalent to a 9% loss in farm revenues (Abolofia, Wilen, & Asche, 2017). Several pest control strategies have been developed over the years, including physical removal technologies (e.g., brushes, water jets and osmolarity shock treatments), feed additives, selective breeding, and chemotherapeutants that are losing their efficacy due to evolved parasitic resistance (Aaen et al., 2015; Torrissen et al., 2013).

Cleaner fish species (wrasse and lumpfish), which actively remove lice from salmon, have been used to delouse farmed Atlantic salmon in sea–cages for several years (Bjordal, 1991) and currently is a widely adopted alternative means of pest control (Johannesen, Joensen, & Magnussen, 2018; Powell et al., 2018). Lumpfish, a native fish to the North Atlantic Ocean, perform well in cold environments (Imsland et al., 2014; Reynolds, Eliassen, Elvergård, Foss, Vikingsstad, & Imsland, 2015) and are the most common farmed and utilized cleaner fish species in the North Atlantic region (Boyce, Ang, & Prickett, 2018). Commercial production of lumpfish has grown exponentially in the last few years (Brooker et al., 2018; Powell et al., 2018), and in Newfoundland, Canada, lumpfish aquaculture has become an emergent industry (Boyce et al., 2018).

Bacterial diseases are the primary challenge for lumpfish delousing performance and survival rates at sea cages (Brooker et al., 2018; Powell et al., 2018). The most frequent bacterial pathogens affecting lumpfish are *Vibrio anguillarum* and *Aeromonas salmonicida* (Gulla, Sorum, Vagnes, & Colquhoun, 2015; Hedeholm, Blicher, & Grønkjær, 2014; Ronneseth, Haugland, Colquhoun, Brudal, & Wergeland, 2017).

V. anguillarum, a Gram-negative bacterium, is the causative agent of vibriosis, a fatal disease impacting marine finfish aquaculture worldwide (Emmy, 1987; Frans et al., 2011; Naka & Crosa, 2011; Naka et al., 2011; Toranzo & Barja, 1990; Toranzo, Romalde, Magariños, & Barja, 2009). Aquaculture losses due to *V. anguillarum* have been reaching magnitudes as high as 100% (Austin, Austin, Sutherland, Thompson, & Swings, 2005; Larsen, Pedersen, & Dalsgaard, 1994; Reynolds, 1988). *V. anguillarum* was first isolated from infected eels during 1909 (Naka & Crosa, 2011) and since then has been reported in several fish species, including lumpfish (Brooker et al., 2018; Hickey & Lee, 2018). *V. anguillarum* outbreaks in lumpfish have been detected in Scotland, Norway and Canada (Breiland et al., 2016; Gulla et al., 2015; Marcos-Lopez, Donald, Stagg, & Mccarthy, 2013; Powell et al., 2018; Vasquez et al., 2018). Development of preventive vaccines for lumpfish against bacterial infections, including vibriosis, have been identified as a high priority by cleaner fish aquaculture industry (Brooker et al., 2018; Powell et al., 2018).

The first vaccine for finfish was developed in 1942 by Duff (Gudding & Van Muiswinkel, 2013). Since then, utilization of vaccines has positively impacted the aquaculture industry, reducing antibiotics utilization, and increasing finfish production and commercial revenues for both fish producers and pharma companies (Brudeseth et al., 2013; Gudding & Van Muiswinkel, 2013; Muktar & Tesfaye, 2016; Plant & Lapatra, 2011). The most common type of commercial vaccines for the aquaculture industry are heat- or formalin-inactivated microorganisms mixed with adjuvants and delivered by intraperitoneal (i.p.) injection. In contrast, bath or immerse vaccine preparations are mixtures of inactivated microorganisms typically in the absence of adjuvant (Plant & Lapatra, 2011). Vaccines against vibriosis are administered to finfish using different delivery methods, and each vaccine composition should satisfy the immunological requirements of the target fish species. Initial vaccination procedures against vibriosis involved the exposure of heat (Fletcher & White, 1973) or formaldehyde-killed *V. anguillarum* cultures to fish (Rombout, Blok, Lamers, & Egberts, 1986). Vaccines for lumpfish against vibriosis have not been designed, and vaccines designed for other fish species, like salmonids, are beginning to be utilized in cleaner fish aquaculture. Vibrogen-2 is a commercial bacterin formulation designed for salmonid species that contains formalin-inactivated cultures of *V. anguillarum* serotypes O1 and O2, and *Vibrio ordalii*, and is becoming frequently utilized in lumpfish. The objective of the study was to evaluate the efficacy of Vibrogen-2 in lumpfish against a local *V. anguillarum* strain isolated from infected lumpfish. We determined that Vibrogen-2 vaccine reduced *V. anguillarum* tissue colonization and delayed mortality but did not confer immune protection to the immunized *Cyclopterus lumpus* against the i.p. *V. anguillarum* challenge.

2 | MATERIALS AND METHODS

2.1 | Bacterial culture

Vibrio anguillarum J360 (NCBI IDs: Chromosome 1 CP034672; Chromosome 2 CP034673; and plasmid CP034674), serotype O2, isolated from an outbreak in lumpfish at Newfoundland was utilized in this study (Vasquez, Cao, Chakraborty, Gnanagobal, Wescott, Boyce, & Santander, 2018). A single colony of *V. anguillarum* J360 was grown routinely in 3 ml of trypticase soy broth (TSB, Difco) at 15°C in a 16-mm-diameter glass tube and placed in a roller for 24 hr. After growth, 300 µl of the overnight culture was added in 30 ml of TSB media using a 250-ml flask and incubated for 24 hr at 15°C with aeration (180 rpm). The bacterial growth was monitored spectrophotometrically until O.D. 600 nm ~0.7 (1×10^8 CFU/ml) using the Genesys 10 UV spectrophotometer (Thermo Spectronic, Thermo Fischer Scientific Inc.). Then, the bacterial culture was centrifuged at 6,000 rpm at room temperature for 10 min. The pellet was washed twice with phosphate-buffered saline (PBS: 136 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4 (pH 7.2); Sambrook & Russell, 2001) and centrifuged at 3.5 *g* at 15°C for 10 min and finally resuspended in 300 µl of PBS (~2.3×10^{10} CFU/ml). The concentrated bacterial inoculum was serial diluted and quantified by plating onto TSA.

2.2 | Fish holding

The experiments were performed in accordance with the guidelines of the Canadian Council on Animal Care and approved by Memorial University of Newfoundland's Institutional Animal Care Committee (protocols #18-01-JS; #18-02-JS). Juvenile specimens of lumpfish 2.9 ± 0.8 g (mean ± *SE*) were obtained from the Dr. Joe Brown Aquatic Research Building (JBARB) at the Department of Ocean Sciences, Memorial University of Newfoundland, Canada.

The animals were kept in 500-L tanks, with flow-through (75 L/min) of U.V.-treated sea water (8–10°C), ambient photoperiod (winter–spring) and 95%–110% oxygen saturation. Biomass density was

maintained at 1–16 kg/m^3 start-to-finish. The fish were fed daily using a commercial diet (Skretting—Europa 15: crude protein (55%), crude fat (15%), crude fibre (1.5%), calcium (3%), phosphorus (2%), sodium (1%), vitamin A (5,000 IU/kg), vitamin D (3,000 IU/kg) and vitamin E (200 IU/kg)) with a ration of 0.5% of their body weight per day. Fish length and weight were measured at different time points to determine the specific growth rate (SGR) according to the formula: SGR = ([ln(final weight (g)) − ln(initial weight (g))] × 100) time $(days)^{-1}$) (Hopkins, 1992).

2.3 | Fish immunization

Lumpfish of ~ 2.9 ± 0.8 g were deep-vaccinated with Vibrogen-2 (Elanco) following the manufacturer's instruction. Briefly, the fish were fasted for 48 hr prevaccination. The bath vaccine solution was prepared by mixing 1 L of well homogenize Vibrogen-2 vaccine solution and 9 L of sea water (1:10 dilution). According to the manufacturer, this dip vaccine suspension can be utilized for up to 100 kg of fish and be reutilized up to 20 times. Two groups of 125 lumpfish each (~500 g per group) were netted and immersed in the vaccine suspension for 30 s. Two mock control-immunized groups of 125 lumpfish were immersed for 30 s in sea water. After 4 weeks post-primary immunization, lumpfish of ~5.2 ± 3.9 g were bath-boosted using a similar process as described previously. After 8 weeks post-primary immunization, lumpfish of ~10 ± 3.3 g were intraperitoneally (i.p.) boosted with 100 μl of Vibrogen-2 vaccine preparation. The control groups were i.p. mock-immunized with 100 μl of PBS. In all cases, food was returned 24 hr post-immunization. Weight and length were monitored weekly to determine the specific growth rate. Additionally, the vaccine preparation was evaluated for bacterial integrity.

2.4 | Vaccine pre-evaluation

Presence and integrity of bacterial cells were assessed by Gram (Leboffe & Pierce, 2015), 5-(4,6-dichlorotriazinyl) aminofluorescein (DTAF; Sigma; Bhupathiraju, Hernandez, Landfear, & Alvarez-Cohen, 1999) and 4′,6-diamidino-2-phenylindole (DAPI; Thermo Fisher) stains. The preparations were observed through light microscopy (Olympus CX21) and confocal microscopy (Nikon AR1).

2.5 | Challenge assays

The infection procedures were done at the AQ2 biocontainment Cold-Ocean Deep-Sea Research Facility (CDRF) under Institutional Animal Care Committee approved protocols (protocol #18-02-JS). Twenty-six weeks post-primary immunization, the fish were transferred from the JBARB to the AQ2 biocontainment laboratory and acclimated for 1 week under similar previously described conditions. After this period, the fish (~108 ± 39 g) were i.p-challenged with 10 or 100 times the *V. anguillarum* J360 LD_{50} dose (2.3×10^5 CFU/dose; Cao et al., 2018). Mortality was monitored daily. The relative per cent of survival (RPS) of vaccinated fish was calculated according to the formula: RPS = [1 − (% vaccinated mortality/% control mortality)] × 100 (Amend, 1981).

2.6 | Fish tissue sampling and analysis

The fish were netted and immediately killed with an overdose of MS222 (400 mg/L; Syndel Laboratories). Liver, spleen, head kidney and brain were aseptically removed at 10 days post-*V. anguillarum* challenge. Sections of the collected tissues were placed into homogenizer sterile bag (Nasco Whirl-Pak®, USA) and weighed, and PBS was added to complete a final volume of 1 ml (weight:volume). Then, the tissues were homogenized, and the suspensions were serially diluted (1:10), and plate counted onto TSA. The plates were incubated at 15°C for 3 days to determine the number of *V. anguillarun* CFU per g of tissue. The total bacteria were normalized to 1 g of tissue according to the initial weight of the tissue; (CFU/g = ([(calculated bacterial cells (CFU/ml) × original tissue weight (g))/1 ml] × 1 g)/original tissue weight (g).

Additionally, tissue sections were fixed in 10% formalin diluted in PBS for three days at room temperature. After this period, the formalin was removed, and the fixed tissues were preserved in PBS at 4°C until en block processing according to standard procedures (Chandler & Roberson, 2009). Sectioned tissues in slides were stained with haematoxylin and eosin (Leica Biosystems) and visualized under the light microscope (Olympus CX21).

2.7 | Statistical analysis

All data are shown as the mean ± standard error (*SE*). Assumptions of normality and homogeneity were tested for detected variances. A one-way ANOVA was used to determine significance followed by Tukey's post hoc test. Differences were considered significant at $p < 0.05$. All statistical analyses were performed using GraphPad Prism 7.0 (GraphPad Software). Kaplan–Meier estimator was used to obtaining survival fractions after the challenges, and to determine the differences between treatments, the log-rank test was used.

3 | RESULTS

3.1 | Vaccine integrity evaluation

Before utilization, Vibrogen-2 was evaluated for bacterin integrity. The Gram, DTAF and DAPI stains showed the presence of intact Gram-negative bacteria, including the presence of bacterial DNA (Figure 1). DTAF has affinity towards proteins, carbohydrates and polysaccharides (Li, Dick, & Tuovinen, 2003; Russ, Zielbauer, Koynov, & Vilgis, 2013), indicating the presence of intact bacterial membrane around the bacterial genomic DNA (Figure 1).

3.2 | Effect of immunization on fish health

The immunization did not influence fish-specific growth rate compared to the mock-immunized groups (Figure S1). Gross pathology examination showed that Vibrogen-2 did not cause tissue adhesion, pigmentation and vaccine remnants at the different time points

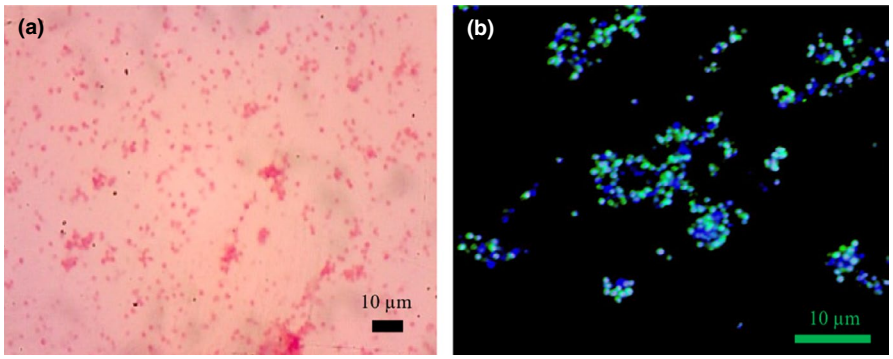

FIGURE 1 Characterization of the commercial vaccine Vibrogen-2. (a) Gram staining indicating the presence of Gram-negative bacteria; (b) 5-(4,6-dichlorotriazinyl) aminofluorescein (DTAF) and 4′,6-diamidino-2-phenylindole (DAPI) staining. DTAF stained the bacterial outer membrane green and DAPI stained the bacterial DNA blue

post-vaccination, compared to the control group. These results suggest that Vibrogen-2 does not cause adverse side effect.

3.3 | Effect of immunization on fish survival post-challenge

PBS-injected control and vaccinated lumpfish were challenged with 2.3×10^6 CFU/dose ($10 \times LD_{50}$) and 2.3×10^7 CFU/dose ($100 \times LD_{50}$) of *V. anguillarum* J360. After challenge, 93.4% of the control group infected with 2.3×10^6 CFU/dose died within 25 days, and 98.4% of the control group infected with 2.3×10^7 CFU/dose died within 11 days (Figure 2). Significant differences ($p < 0.0001$) were found between control groups infected with low (2.3×10^7 CFU/dose) and high (2.3×10^7 CFU/dose) dose of *V. anguillarum*.

After 40 days post-challenge, 86% of the vaccinated group challenged with 2.3×10^6 CFU/dose died, and 97.4% of the vaccinated group challenged with 2.3×10^7 CFU/dose died (Figure 2). No significant differences were found between vaccinated groups challenged with low and high dose of *V. anguillarum*. However, significant differences were found between control and vaccinated groups ($p < 0.0001$; Figure 2).

The RPS of vaccinated fish was 8% for the group challenged with the low dose (2.3×10^6 CFU/dose) and 1% for the group challenged with the high dose (2.3×10^7 CFU/dose).

3.4 | *Vibrio anguillarum* tissue colonization

V. anguillarum loads were quantified in liver, spleen, head kidney, brain and blood in vaccinated and naïve (control) lumpfish at 10 days post-challenge. Naïve lumpfish infected with 2.3×10^7 CFU/dose of *V. anguillarum* showed high levels of bacterial counts in liver, spleen, head kidney, brain and blood, in all sampled fish (Figure 3). In contrast, in naïve lumpfish infected with the lower dose of *V. anguillarum* (2.3×10^6 CFU/dose), the bacterium was not detected in all sampled individuals (Figure 3).

In contrast to infected control fish, *V. anguillarum* was not detected in vaccinated fish challenged with the lower dose of pathogenic bacteria (2.3×10^6 CFU/dose), and significant lower bacterial loads were detected in vaccinated fish challenged with the higher dose of *V. anguillarum* (2.3×10^7 CFU/dose) at 10 days post-challenge (Figure 3).

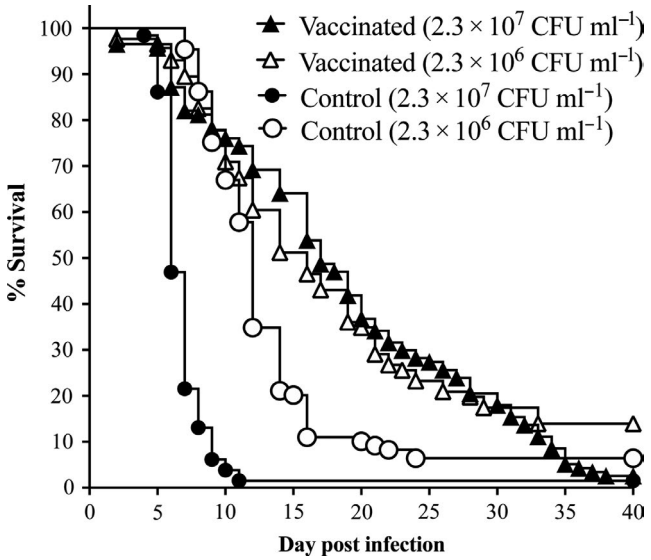

FIGURE 2 Survival of vaccinated and control fish after *Vibrio anguillarum* J360 challenge. Lumpfish were challenged with 2.3×10^6 ($10 \times LD_{50}$) and 2.3×10^7 ($10 \times LD_{50}$) CFU/dose of *V. anguillarum* J360. Significant differences were detected between control groups and between control groups and vaccinated groups. No significant differences were detected between vaccinated groups ($p < 0.0001$)

3.5 | Histopathology

The histopathological analysis correlates with the mortality and bacterial colonization results (Figures 3 and 4). In contrast to non-infected fish, acute inflammation (neutrophil and fibrin exudate formation) and necrosis were observed in spleen, liver and head kidney in infected fish (Figure 4). Intracellular *V. anguillarum* was detected in spleen and head kidney of infected fish (Figure 4).

4 | DISCUSSION

As mentioned previously, vaccines for lumpfish against bacterial infections are a high priority for this emergent aquaculture species. *Vibrio anguillarum* is one of the most recurrent pathogens in lumpfish aquaculture settings (Brooker et al., 2018; Marcos-Lopez, Donald, Stagg, & Mccarthy, 2013). *Vibrio anguillarum* bacterin-based

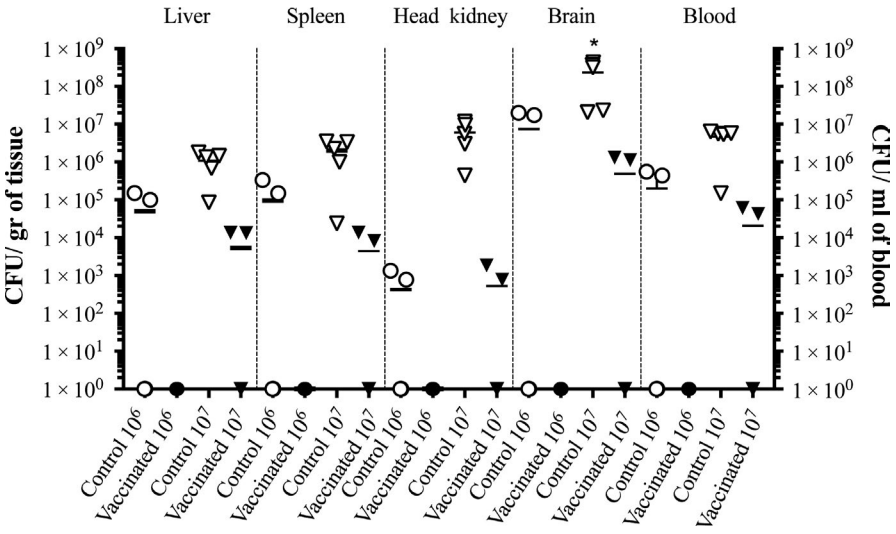

FIGURE 3 *Vibrio anguillarum* tissue colonization in lumpfish at 10 days post-challenge. *Vibrio anguillarum* tissue colonization was determined in liver, spleen, head kidney, brain, and blood of vaccinated and mock immunized control fish (n = 5) i.p. challenged. *V. anguillarum* loads were significantly high in brain of control fish infected with 2.3×10^7 ($10 \times LD_{50}$) CFU/dose of *V. anguillarum* J360 ($p < 0.0001$). The vaccinated fish showed a lower *V. anguillarum* colonization with no detectable bacterial loads in tissues sampled from fish challenged with 2.3×10^6 CFU/dose

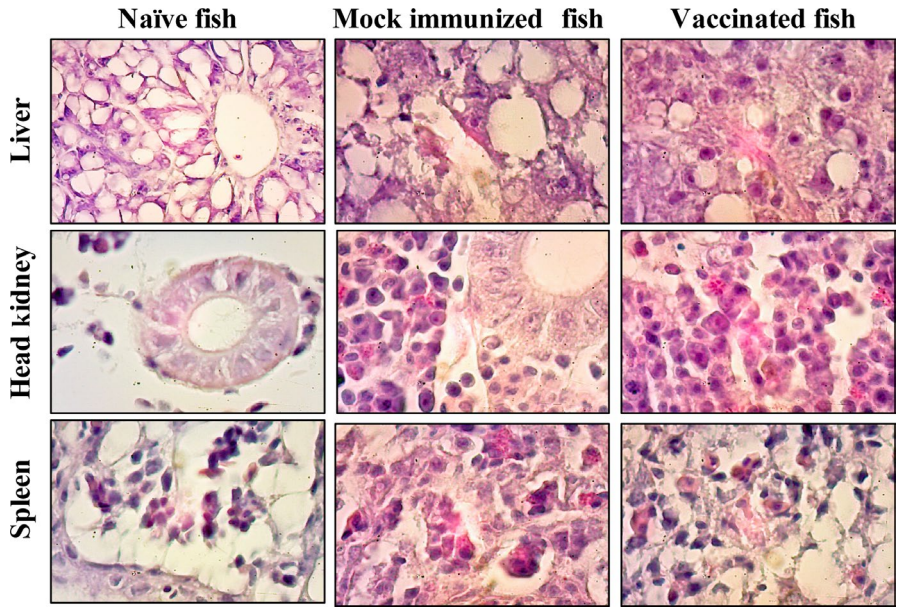

FIGURE 4 Histopathology of lumpfish tissues stained with haematoxylin and eosin (1,000×). Liver, head kidney and spleen were collected from naïve, control and vaccinated lumpfish at 10 days post-challenge with *V. anguillarum* J360 (2.3×10^7 CFU/dose)

commercial vaccines are widely available; however, information about their efficacy in different cold water fish species, like lumpfish, is missing.

Recently, *V. anguillarum* J360 was isolated from an outbreak in lumpfish vaccinated with Vibrogen-2 (2018, & Santander, 22018), raising the question about vaccine efficacy. Here, we evaluate the efficacy of Vibrogen-2 vaccine in lumpfish against a local *V. anguillarum* isolate. We confirm that *V. anguillarum* J360 is highly virulent in lumpfish at 10°C (Figure 2).

Vibrio anguillarum tissue colonization correlates with mortality data (Figures 2 and 3), where naïve-infected fish with the lower dose (2.3×10^6 CFU/dose) at 10 days post-infection have an asymptomatic infection or incubation period. In contrast, at 10 days post-infection, naïve fish infected with the higher dose (2.3×10^7 CFU/dose) showed an evident systemic infection (Figures 3 and 4). Additionally, this correlates with the significant survival differences between lumpfish infected with the low and the high dose of *V. anguillarum* ($p < 0.0001$). Furthermore, infected fish with *V. anguillarum* displayed an active erratic swimming, which correlated with the high loads of bacteria found in the brain (Figure 3).

Survival at post-challenge of vaccinated fish suggests that immunization with Vibrogen-2 delays mortality for about 2 weeks but did not confer immune protection (Figure 2). Three main factors seem to influence vaccine efficacy against *V. anguillarum*, including temperature, fish species and vaccine preparation. Recently, it has been shown that at lower temperatures lumpfish immune response to vaccination is low (Erkinharju, Dalmo, Vågsnes, Hordvik, & Setternes, 2017). In contrast, evaluation of a similar commercial vaccine (AquaVac Vibrio;

V. anguillarum O1 and O2 without adjuvants) with similar delivery method (bath and i.p. boost) protects European sea bass (*Dicentrarchus labrax*) against the i.p. *V. anguillarum* challenge (~10^6 CFU/ml) at 17–20°C (Galeotti et al., 2013), suggesting that temperature could be an important factor to consider for lumpfish vaccination and perhaps a different delivery strategy should be also considered.

In the context of vaccine formulation, it has been suggested that the *V. anguillarum* serotype is also an important immune protective factor in vaccine preparations; however, *V. anguillarum* J360 is an O2 serotype that is included in Vibrogen-2 vaccine. It has been shown that a single injection of heat-inactivated *V. anguillarum* bacterin was effective in chinook salmon (*Oncorhynchus tshawytscha*), in contrast to a formalin-inactivated *V. anguillarum* bacterin (Anitipa, 1976). However, similar vaccine preparations were not effective in coho salmon (*O. kisutch*; Anitipa, 1976). In another study, immersion and i.p. immunization with a formalin-killed *V. anguillarum* protected Atlantic halibut (*Hiippoglossus hippoglossus* L.) against the *V. anguillarum* challenge (1×10^7 CFU/ml; Bowden, Menoyo-Luque, Bricknell, & Wergeland, 2002), suggesting that *V. anguillarum* vaccines are not effective across fish species and perhaps specific vaccine preparations are required for lumpfish.

The natural route of *V. anguillarum* infection is likely through mucosa(e.g., skin and orogastric infection) and skin lesion (Hickey & Lee, 2018), and although Vibrogen-2 did not conferred immune protection against the i.p. challenge, this vaccine is still a useful tool to delay natural outbreaks.

In summary, the commercial vaccine Vibrogen-2 delayed mortality in about three weeks compared to PBS-injected control fish but did not confer immune protection to *C. lumpus* against a local *V. anguillarum* strain.

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CONFLICT OF 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.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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