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ORIGINAL ARTICLE

Antibacterial treatment of lumpfish (*Cyclopterus lumpus*) experimentally challenged with *Vibrio anguillarum*, atypical *Aeromonas salmonicida* and *Pasteurella atlantica*

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Abstract

Lumpfish is a novel farmed species used as cleaner fish for the removal of lice from farmed salmon. As often with new, farmed species, there are challenges with bacterial infections. The frequency of prescription of antibiotic agents to lumpfish is increasing, despite the lack of knowledge about appropriate doses, duration of treatment and application protocols for the various antibacterial agents. In the current study, we have tested the effect of medicated feed with florfenicol (FFC), oxolinic acid (OA) and flumequine (FLU) on lumpfish experimentally challenged with *Vibrio anguillarum*, atypical *Aeromonas salmonicida* and *Pasteurella atlantica*. We found that all three antibacterial agents efficiently treated lumpfish with vibriosis using 10 and 20 mg kg^{-1} day^{-1} of FFC, 25 mg kg^{-1} day^{-1} of OA and 25 mg kg^{-1} day^{-1} FLU, whereas only FFC (20 mg kg^{-1} day^{-1}) had good effect on lumpfish with pasteurellosis. None of the antibacterial agents were efficient to treat lumpfish with atypical furunculosis. FFC 20 mg kg^{-1} day^{-1} showed promising results in the beginning of the experiment, but the mortality increased rapidly 14 days post-medication. Efficient treatment is important for the welfare of lumpfish and for reducing the risk of development of antibiotic-resistant bacteria. To our knowledge, this is the first study to establish protocols for antibacterial treatment of lumpfish.

KEYWORDS

atypical furunculosis, florfenicol, flumequine, oxolinic acid, pasteurellosis, vibriosis

1 | INTRODUCTION

Lumpfish (*Cyclopterus lumpus* L.), also known as lumpsucker, are now farmed and used as cleaner fish to remove sea lice from farmed Atlantic salmon in Europe and Canada (Powell et al., 2018; Treasurer, 2018; Haugland et al., 2020). In Norway, the number of farmed lumpfish has increased rapidly in recent years and 34 million lumpfish were deployed in 2020 (http://www.fiskeridir.no). High mortality among lumpfish has been reported, most caused by bacterial infections with atypical *Aeromonas salmonicida*, *Vibrio* spp., *Pasteurella* sp. or *Pseudomonas anguilliseptica* (Scholz et al., 2018; Erkinharju, Dalmo, et al., 2021; Erkinharju, Grønbech et al., 2021). The *Pasteurella* sp. isolates from lumpfish have recently been named *Pasteurella atlantica* (Ellul et al., 2021; Erkinharju, Grønbech et al., 2021). Atypical and

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typical *A. salmonicida*, major pathogens of diverse fish species found in both marine and freshwater environments (reviewed in Wiklund & Dalsgaard, 1998; Menanteau-Ledouble et al., 2016), can be divided into 14 subgroups, based on variation in the gene (*vapA*) encoding the A-layer protein (Gulla et al., 2016). *A. salmonicida* is a highly diverse group of bacteria, both regards to biochemical characteristics, growth conditions and extracellular proteases, and includes several atypical strains. Pathogenic bacteria for lumpfish are dominated by atypical *A. salmonicida* isolates belonging to A-layer group VI. Among *Vibrio* spp., *V. anguillarum* is the major pathogen, but other *Vibrio* species have also caused mortality, including *V. ordalii*, *V. tapetis*, *V. logei*, *V. wodanis* and *V. splendidus* (Erkinharju, Dalmo, et al., 2021; Erkinharju, Grønbech et al., 2021; Haugland et al., 2020). Except for *V. ordalii*, it is debated whether these are primary or secondary pathogens. Furthermore, *P. atlantica* causing systemic bacterial infection in lumpfish has become a serious infection problem (Alarcon et al., 2016; Ellul, Walde, et al., 2019; Ellul et al., 2021). Phenotypically and histopathologically, the *P. atlantica* isolates from lumpfish are similar, but not identical, to *Pasteurella* spp. isolated from farmed Atlantic salmon in Norway and *Pasteurella skyensis* in Scotland (Alarcon et al., 2016; Ellul, Walde, et al., 2019; Ellul et al., 2021).

Efforts to develop vaccines to protect against bacterial diseases in lumpfish are ongoing (Rønneseth, Brudal, et al., 2017; Rønneseth, Haugland, et al., 2017; reviewed in Haugland et al., 2018; Haugland & Rønneseth, 2018, Ellul, Walde, et al., 2019, Chakraborty et al., 2019; Erkinharju et al., 2017; 2018; 2019). In Norway, lumpfish are vaccinated by intraperitoneal (i.p.) injection against vibriosis and atypical furunculosis when they have reached a minimum size of 8 g (Haugland et al., 2018). The vaccines protect against vibriosis, but atypical furunculosis is still a problem in farmed lumpfish. Atypical *A. salmonicida* is a heterogenic group, and it is difficult to identify one isolate that protects against all subtypes (Nordstrand et al., 2017). The vaccines currently available do not include *P. atlantica*, one of the major reasons being the fastidious growth requirements of this bacterium. Recently, Ellul, Walde, et al. (2019) and Ellul, Bulla, et al. (2019) were able to cultivate *P. atlantica* in broth media to a density of 2×10^9 bacteria ml^{-1} and establish a bath challenge model (Ellul, Walde, et al., 2019). Two R&D vaccines against pasteurellosis have been tested, but despite high levels of specific antibodies being raised, the vaccines did not provide sufficient protection against disease (Ellul, Bulla, et al., 2019). At early life stages (<8 g), immersion vaccination can be performed, but it is not yet known at what size/age the lumpfish is immunocompetent (Rønneseth, Haugland, et al., 2017; Chakraborty et al., 2019). As bacterial diseases affect lumpfish in all stages of the production, treatment by antibacterial agents is often the only option for juvenile fish. Lumpfish in sea pens with salmon are not treated with antibacterial agents, and development of efficient vaccines is therefore crucial.

Despite progress in development of immune prophylactic measures to lumpfish (Haugland et al., 2018; Ellul, Bulla, et al., 2019), the number of treatments with antibacterial agents is still high (Grave et al., 2019). Excessive or improper use of antibacterials may lead to development of resistance in bacterial pathogens. In recent years, there has been an increased understanding of the mechanisms of antimicrobial resistance (Miranda, Tello et al., 2013; Blair, Webber et al., 2015; Munita & Arias, 2016) and the use of antibiotics in aquaculture is a worldwide concern. Antibacterials currently authorized for use in salmon aquaculture vary between countries, where oxytetracycline, florfenicol (FFC), trimethoprim/sulpha derivatives and quinolones are among the most widely used substances (Cabello et al., 2013). Oxolinic acid (OA) and florfenicol have shown high efficacy in treating several bacterial diseases in Atlantic halibut (*Hippoglossus hippoglossus* L.), corkwing wrasse (*Symphodus melops* L.), Goldsinny wrasse (*Ctenolabrus rupestris* L.), turbot (*Scophthalmus maximus* L.), Atlantic cod (*Gadus morhua* L.) and Atlantic salmon (*Salmo salar* L.) (Samuelsen, 1997; Samuelsen et al., 1998; Samuelsen et al., 1999; Samuelsen et al., 2000; Samuelsen et al., 2002; Samuelsen, 2003; Samuelsen et al., 2003). In Norway, medicated feeds containing FFC (0.83 and 2 g FFC per kg feed) and OA (5 g OA per kg feed) are commercially available for lumpfish. However, efficacy studies have not yet been reported. Prior to initiating treatment of diseased fish, it is important to know the sensitivity of the pathogen for the antibacterial agent considered for use. Knowledge of the sensitivity and minimum inhibitory concentration (MIC) value can, together with pharmacokinetic data, be used to calculate a theoretical concentration of an antibacterial needed to achieve an effective treatment (Kverme et al., 2019; Haugland et al., 2019). It is, however, of major importance that the efficacy of antibacterial treatments is experimentally verified. Kverme et al. (2019) uncovered some isolates of *A. salmonicida* with reduced sensitivity towards OA, but high sensitivity to flumequine (FLU). Furthermore, Haugland et al. (2019) showed that FLU has advantageous pharmacokinetic properties in lumpfish compared with OA. Thus, the current study was initiated to evaluate the efficacy of FFC, OA and FLU in treating experimentally induced infections with *V. anguillarum*, atypical *A. salmonicida* and *P. atlantica* in lumpfish.

2 | MATERIALS AND METHODS

2.1 | Experimental fish

Unvaccinated, farmed lumpfish (*C. lumpus*) were supplied by Vest Aqua Base AS, a commercial breeder in Vestland County, Norway. Head kidney samples from 15 to 20 fish were screened for *V. anguillarum* O1, *V. anguillarum* O2, atypical *A. salmonicida* and *Pasteurella* sp. at PHARMAQ Analytiq (a diagnostic laboratory) by real-time RT-PCR. All samples were negative. The fish were kept in tanks (500 L) at the Aquatic and Industrial Laboratory (ILAB), Bergen, Norway. The water temperature was 12°C, the salinity 34 ‰ and the light regime 12 h light: 12 h dark. The water flow was 300–400 L per hours per tank, and the outlet water had a minimum of 77% oxygen saturation. The fish were fed with the commercial dry feed Gemma Silk (Experiment 1 and Experiment 2) and Clean Assist (Experiment 3), 1.2- to 1.5-mm pellets (both feeds from Skretting). When the fish were transferred to the challenge unit at ILAB, they were 36.2 ± 10.6 g and 8.5 ± 0.7 cm. The animal experiments were

approved by the Norwegian Animal Research Authority under the identification codes FOTS-ID: 10178 and 14129. Permissions to use OA and to use and make feed with FLU were approved by the Norwegian Medicines Agency.

2.2 | Medical feed

Both the FFC and OA medicated feeds, Gemma Silk, 1.5-mm pellets (Experiment 1 and Experiment 2), and Clean Assist feed, 1.5-mm pellets (Experiment 3), were commercial feeds for lumpfish (Skretting). The medicated feed containing FLU was made in the laboratory by coating the 1.5-mm pellets (Gemma Silk in Experiment 1 and Experiment 2, and Clean Assist in Experiment 3) with a premix of D-glucose and FLU as described previously (Haugland et al., 2019). Fish in the control group (infected, non-treated group) got regular, non-medicated feed (same pellet size and brand as the treated groups).

2.3 | Bacteria grown for experimental challenge

Vibrio anguillarum serotype O1 isolated from diseased lumpfish was cultured in tryptic soy broth (TSB) (Becton, Dickinson and Company) supplemented with 1.5% NaCl at 20°C, 200 rpm to late exponential phase. The bacteria were washed once with PBS (BioWhittaker) and diluted to 2×10^3 cells ml^{-1} using a CASY cell counter (Innovatis). Atypical *A. salmonicida* isolated from diseased lumpfish was grown in TSB at 18°C, 200 rpm to late exponential phase. The bacteria were washed once with PBS, and the bacteria number was determined by CASY cell counter and diluted to 1×10^5, 2×10^4 and 4×10^3 cells ml^{-1}. The *P. atlantica* isolated from diseased lumpfish was grown in TBS supplemented with 1.5% NaCl and 10% foetal bovine serum (FBS, Australian origin; Gibco Life Technologies) as described previously (Ellul, Walde, et al., 2019). The bacteria were washed once with PBS, counted and diluted as described for atypical *A. salmonicida*.

2.4 | Bacteria grown for sensitivity testing and MIC determination

Vibrio anguillarum and atypical *A. salmonicida* were grown to late exponential phase in Mueller Hinton Broth 2 (MHB2), cation-adjusted (Sigma-Aldrich). *P. atlantica* was grown in MHB2 supplemented with 10% FCS. All bacteria were grown at 22°C, 200 rpm. The bacteria were diluted to 5×10^6 bacteria ml^{-1}, and 100 µl was added to each well in 96-well round-bottom microtest plates (Sarstedt). FFC, FLU and OA (512–0.001 µg/ml) were included (twofold dilutions, three parallels of all dilutions). Controls with only bacteria, no antibiotics and growth medium only were included on each plate. The plates with *V. anguillarum* were incubated at 22°C for 24 h, while plates with atypical *A. salmonicida* and *P. atlantica* were incubated for 48 h. The MIC was determined after visual inspection, given as the concentration where no growth could be observed.

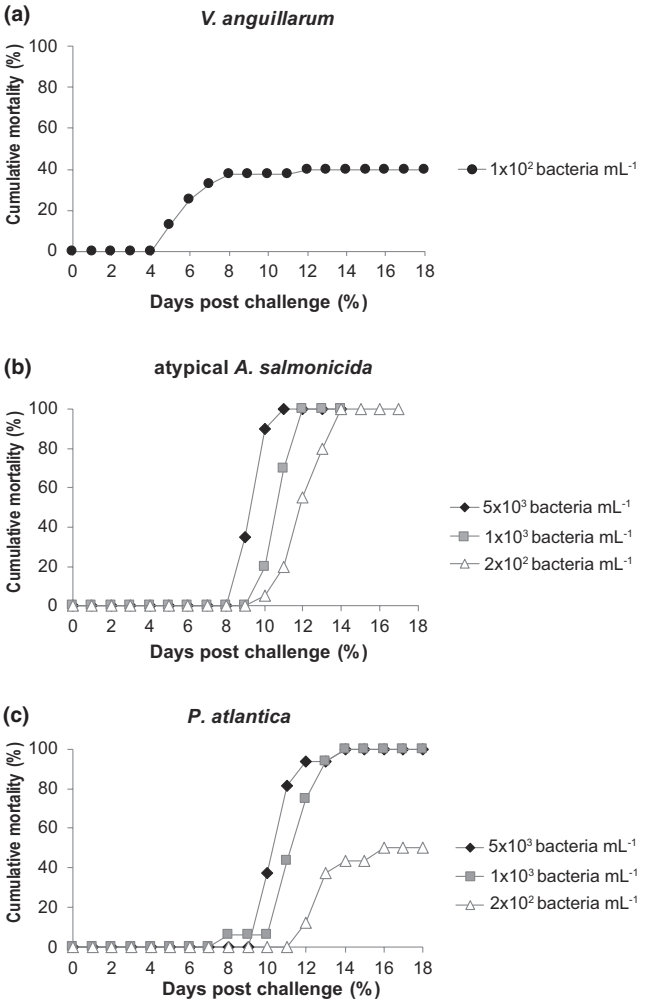

FIGURE 1 Mortality curves of lumpfish following the bacterial challenge. (a) Cumulative mortality of lumpfish after challenge with 1×10^2 *Vibrio anguillarum* per fish. (b) Cumulative mortality of lumpfish after challenge with 5×10^3, 1×10^3 and 2×10^3 atypical *Aeromonas salmonicida* per fish. (c) Cumulative mortality of lumpfish after challenge with 5×10^3, 1×10^3 and 2×10^3 bacteria *Pasteurella atlantica*

2.5 | Determination of challenge dose

Fifty µl/fish of the three doses (5×10^3, 1×10^3 and 2×10^2 bacteria) of atypical *A. salmonicida* ($n = 16$ per group) and *P. atlantica* ($n = 20$ per group) was given by i.p. injection to separate groups of fish. Based on cumulative mortality and time of onset of mortality for each dose of pathogen (Figure 1a), the most appropriate challenge doses and time point to start medication after challenge were determined. Initially, a dose of 1×10^2 bacteria ml^{-1} of *V. anguillarum* was used ($n = 20$ fish per tank in two tanks), based on previous experiments (Rønneseth et al., 2014). However, using this dose, the mortality started after 5 days and the cumulative mortality reached only 40% (Figure 1a), which was considered low for a control group. Therefore, in the main experiment, the concentration of *V. anguillarum* in the suspension was increased from 1×10^2 bacteria to 2×10^2 bacteria and the medication started 2 days post-challenge

(dpc). Based on the preliminary results, a dose of 2×10^2 bacteria per fish and medication starting 5 dpc were chosen for atypical *A. salmonicida* (Figure 1b). For *P. atlantica*, a dose of 5×10^2 bacteria per fish and starting medication 5 dpc were decided (Figure 1c).

2.6 | Experimental challenge and treatment experiment

For each of the experiments 1, 2 and 3 (see below), four hundred and eighty fish were transferred to the challenge rearing unit one week prior to challenge for acclimatization. The fish were randomly divided into sixteen groups of 30 fish per 150-L tanks: three parallel tanks for each of the four treatment regimens (Tables 1 and 2) and four tanks containing infected, non-treated control fish. The water had a temperature of 12°C, salinity of 34 ‰ and a flow rate of 6 L min^{-1}. The light regime was 12 h light:12 h dark.

Experiment 1 (*V. anguillarum*)

The fish were challenged i.p. with 50 µl *V. anguillarum* bacterial suspension (2×10^2 bacteria) and the medication started 2 dpc.

Experiment 2 (atypical *A. salmonicida*)

The fish were challenged i.p. with 50 µl atypical *A. salmonicida* (2×10^2 bacteria), and the medication started 5 dpc.

Experiment 3 (*P. atlantica*)

The fish were challenged i.p. with 50 µl *P. atlantica*. (5×10^2 bacteria), and the medication started 5 dpc. The antibacterial agents and doses administered for each experiment are listed in Table 1 for experiments 1 and 2 and in Table 2 for Experiment 3. It has been speculated that *P. atlantica* might be a facultative intracellular bacterium (Ellul, Walde, et al., 2019). Therefore, based on previous experience with treating *Fransicella noatunensis* infection in cod (Samuelsen, unpublished results), the doses and treatment chosen in Experiment 3 differed from those used in experiments 1 and 2. In addition to medicated feed, the fish were given non-medicated feed in all three experiments to maintain a total daily feeding rate of 1.5% of the body weight.

Each morning, the fish tanks were cleaned, and a strainer was placed over the drain grate allowing excess feed to be collected. The excess feed from each tank was dried at 90°C for 18 h and the amount of feed consumed calculated using the factor of 1.1, that is the ratio of dried versus non-dried feed (Kverme, 2017).

2.7 | Macroscopic examination and bacteria sampling

Tissues and skin of moribund fish from all experiments were examined macroscopically. From dead and moribund fish, bacteria were isolated from blood, head kidney, spleen, liver and heart using a graft needle, and streaked onto agar plates. Samples from fish challenged with *V. anguillarum* were plated onto TSA plates with 2% NaCl and plates with *Vibrio*-specific medium containing thiosulphate–citrate–bile salts–sucrose agars (Sigma). Samples from fish challenged with atypical *A. salmonicida* were plated onto TSA plates. Single colonies were further streaked onto blue agar plates (TBS containing 0.01% Coomassie Brilliant Blue R-250) to examine whether reisolated atypical A. salmonicida still contained A-layer. Samples from fish challenged with *P. atlantica* were streaked onto blood agar plates containing 2% NaCl. The isolated bacteria were identified using 16S rDNA sequencing and universal primers as previously described (Rønneseth, Brudal, et al., 2017a). Furthermore, bacteria isolated from *Vibrio*-infected fish were analysed by an agglutination test (MONO-Va; Bionor Laboratories AS).

TABLE 1 Treatment regimen of lumpfish after experimental challenge with *Vibrio anguillarum* (Experiment 1) and atypical *Aeromonas salmonicida* (Experiment 2)

Feed		Conc.	Daily dose (mg/kg)	Total dose during treatment (mg/kg)	Amount of medical feed (% of body weight)[a]	Days of treatment post-infection
Floraqpharma vet.	FFC10	0.83g/kg	10	100	1.0%	Daily, dpc 2–11 (*V. ang*) Daily, dpc 5–14 (atyp *A. sal.*)
Floraqpharma vet.	FFC20	2g/kg	20	200	1.0%	Daily, dpc 2–11 (*V. ang*) Daily, dpc 5–14 (atyp *A. sal.*)
Oxolinic acid vet.	OA25	5g/kg	25	150	0.5%	Dpc 2, 3, 5, 7, 9, 11 (*V. ang*) Dpc 5, 6, 8,10, 12, 14 (atyp *A. sal.*).
Flumequine	UB25	5g/kg	25	150	0.5%	Dpc 2, 3, 5, 7, 9, 11 (*V. ang*) Dpc 5, 6, 8, 10, 12, 14 (atyp *A. sal.*).

[a]Total amount of medical and non-medical feed was 1.5% of body weight.

TABLE 2 Treatment regimen of lumpfish after experimental challenge with *Pasteurella atlantica* (Experiment 3)

Feed		Conc.	Daily dose (mg/kg)	Total dose during treatment (mg/kg)	Amount of medical feed (% of body weight)[a]	Days of treatment
Floraqpharma vet.	FFC20	2g/kg	20	200	1.0%	Daily, dpc 5–14
Floraqpharma vet.	FFC20	2g/kg	20	300	1.0%	Daily, dpc 5–19
Oxolinic acid vet.	OA25	5g/kg	25	150	0.5%	Dpc 5, 6, 8,10, 12, 14
Oxolinic acid vet.	OA25	5g/kg	25	250	0.5%	Daily, dpc 5–14

[a]Total amount of medical and non-medical feed was 1.5% of body weight

TABLE 3 MIC values in MHB2, cation-adjusted

	Antibacterial agents		
Bacteria	FFC (µg/ml)	OA (µg/ml)	FLU (µg/ml)
Vibrio anguillarum O1	0.5	0.064	0.125
Atypical *Aeromonas salmonicida*	1.0	0.064	0.125
Pasteurella atlantica	0.125	0.5	0.25

2.8 | Statistical analyses

Kaplan-Meier survival analyses were performed using SigmaStat 3.5 (Systat Software Inc., Richmond, USA). Log-rank tests were used to evaluate the effect of medical treatment, and multiple pairwise comparisons were done using the Holm–Sidak method. Differences were considered significant if $p < .05$. Relative percentage survival (RPS) values were calculated according to Inglis et al. (1991).

3 | RESULTS

3.1 | Sensitivity to antibacterials

The sensitivity, specified as MIC values, of *V. anguillarum*, atypical *A. salmonicida* and *P. atlantica* towards FFC, OA and FLU is given in Table 3. *V. anguillarum* and atypical *A. salmonicida* were more sensitive to OA than to FLU, while *P. atlantica* was more sensitive to FLU. Highest sensitivity towards FFC was seen in *P. atlantica* and lowest in atypical *A. salmonicida*.

3.2 | Challenge dose test

Prior to experiments 1, 2 and 3, preliminary studies were performed to determine the most appropriate doses to use for challenge and time to start the medication. The dose of *V. anguillarum* gave onset of mortality at day 5 and a cumulative mortality of 40% (Figure 1a), which were in the lower region for a control group in an effective study. The dose for the final experiment was therefore increased

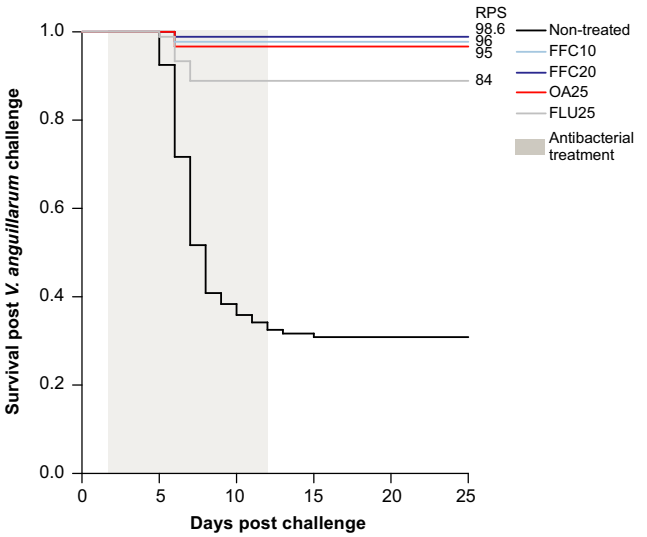

FIGURE 2 Survival curve of lumpfish experimentally challenged with *Vibrio anguillarum* after antibacterial treatments. Black line is non-treated control ($n = 4$ tanks, 30 fish in each tank), light blue line is treatments with FFC 10 mg kg^{-1} day^{-1} for 10 days, dark blue line is treatments with FFC 20 mg kg^{-1} day^{-1} for 10 days, red line is treatments with OA FFC 25 mg kg^{-1} day^{-1} for 6 days, and grey line is treatments with FLU 25 mg kg^{-1} day^{-1} for 6 days (see Table 1 for more details of feeding during the treatment period). Average of three parallel tanks (with 30 fish in each tank) is shown for all treatment groups. RPS (relative percentage survival) is given for each treatment

from 1×10^2 to 2×10^2 bacteria per fish. It was decided to start the medication 2 dpc.

For atypical *A. salmonicida*, mortality started at 9 dpc for the highest dose and 10 dpc for the low and medium dose. However, since all doses gave 100% mortality (Figure 1b), the lowest dose (2×10^2 bacteria per fish) was chosen for Experiment 2 and the start of medication was set to 5 dpc.

The doses of *P. atlantica* gave onset of mortality at days 9, 11 and 12 post-challenge for the high (5×10^3 bacteria per fish), medium (1×10^3 bacteria per fish) and low (2×10^2 bacteria per fish), doses respectively. The cumulative mortality was 100% for the two highest doses, and 50% for the low dose (Figure 1c). It was therefore

decided to use a dose of 5×10^2 bacteria per fish in the main experiment and to start medication 5 dpc.

3.3 | Experiment 1: Treatment of lumpfish after experimental challenge with *V. anguillarum*

Following the challenge with *V. anguillarum*, the mortality started 5 dpc in the non-medicated groups (total of 120 fish, 4 tanks each with 30 fish). The mortality rate was rapid reaching a cumulative mortality of 62% at 9 dpc. At the end of the experiment (25 dpc), the mean cumulative mortality in the 4 control tanks was 68% (Figure S1) However, in one tank, the cumulative mortality was significantly lower (43%) compared with the mean value of the remaining three (77.7%). Medication, started 2 dpc, was very efficient for all the drugs tested resulting in mean cumulative mortalities of 2.2% (FFC10), 1.1% (FFC20), 3.3% (OA) and 11.1% (FLU). The Kaplan–Meier survival analysis (Figure 2) showed a statistically significant effect of all treatment doses and antibacterial agents ($p < .001$). Pairwise comparison between groups showed that all the medicated groups were statistically significantly different from the non-treated control groups. No statistical difference between the medicated groups was found, except for between FFC20 and FLU (Table S1). The RPS values ranged from 84% to 98.6%, reflecting the high efficacy of all the antibacterials tested to treat vibriosis in lumpfish.

Macroscopic observation of moribund fish had swollen gut and ascites in the abdominal cavity (Figure 3a). Identification of *V. anguillarum* as the causative pathogen was verified by the *Vibrio* agglutination test and growth on *Vibrio* selective agar (TCBSA) (Figure 3b).

3.4 | Experiment 2: Treatment of lumpfish after experimental challenge with atypical *A. salmonicida*

Atypical *A. salmonicida* used for challenge was grown at 18°C since it may not express the A-layer surface protein at higher temperatures. The A-layer is important for virulence, and an SDS-PAGE was performed to ensure that the A-layer was present (Figure 5a). Mortality in the challenged, non-medicated groups (total of 120 fish, 4 tanks each with 30 fish) started 10 dpc and had reached 98.3% 17 dpc. The medication started at 5 dpc, and high mortalities were seen in the groups of fish given FFC10, OA and FLU (Figure S2). In the FFC20 groups, a lower mortality was observed until 30 dpc (14 days after terminated medication). However, between 30 and 40 dpc, the mortality rate increased significantly, and cumulative mortality reached 82.2% at the end of the experiment (Day 48) (Figure S2). According to the Kaplan–Meier survival analysis, all the treatments were significantly different from the non-treated group (Figure 4, Table S2). However, the RPS values were low and ranged from 7% to 23%, indicating that none of the tested antibacterials and doses efficiently treated atypical furunculosis infection in lumpfish.

Macroscopic observations of moribund fish were swollen gut (Figure 5b) and white nodules in head kidney, liver and other internal organs (Figure 5c,d). To investigate whether atypical *A. salmonicida* reisolated from the fish had A-layer, a single colony was streaked onto a blue agar plate. Blue colour verified the presence of A-layer (Figure 5e).

3.5 | Experiment 3: Treatment of lumpfish after experimental challenge with *P. atlantica*

Based on results in Experiment 2 (Figure 4), and the indications that *P. atlantica* might be a facultative intracellular bacterium, we chose to compare the treatment of FFC 20 mg kg^{-1} day^{-1} for 10 and 15 days, and OA 25 mg kg^{-1} day^{-1} for 6 days (5, 6, 8, 10, 12 and 14 dpc) and 10 days. Mortality in the challenged, non-medicated groups (total of 120 fish, 4 tanks each with 30 fish) started at 10 dpc. The mortality rate in the control groups was high, and cumulative mortality had reached 71.7% after 5 days and 76.7% at the end of the experiment. A low mortality rate was obtained for both medication regimens with FFC (FFC20 daily for 10 and 15 days) (Figure S3). However, the fish treated for 10 days were less active and had a darker skin pigmentation than fish treated for 15 days. For fish medicated with OA, a major difference in cumulative mortality was observed between the groups treated for 10 days with 22.2% mortality and those treated for 6 days with 54.4% mortality. Results from the Kaplan–Meier survival analysis are summarized in Figure 6 and Table S3, showing a significant effect of all the treatment regimens, and also statistical differences between FFC and OA. The RPS values for florfenicol were found to be 95.7% for the 10-day medication regimen and 97% when treated for 15 days. A major difference in RPS was found using OA where daily administration of 25 mg/kg for 10 consecutive days (total dose 250 mg/kg) gave an RPS value of 71%, whereas 6 administrations over a 10-day period (total dose 150mg/kg) were much less efficient with an RPS value of 29.1%.

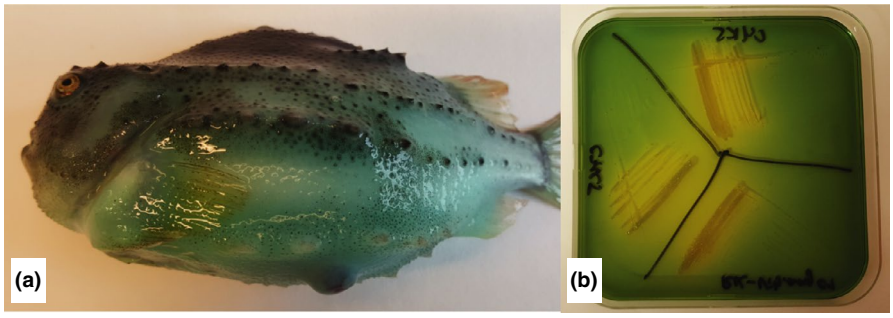

FIGURE 3 Macroscopic observations of diseased lumpfish with vibriosis. (a) Swollen anus and ascites in the abdominal cavity and (b) bacteria growing on agar plates with *Vibrio* selective medium containing thiosulphate–citrate–bile salts–sucrose agar

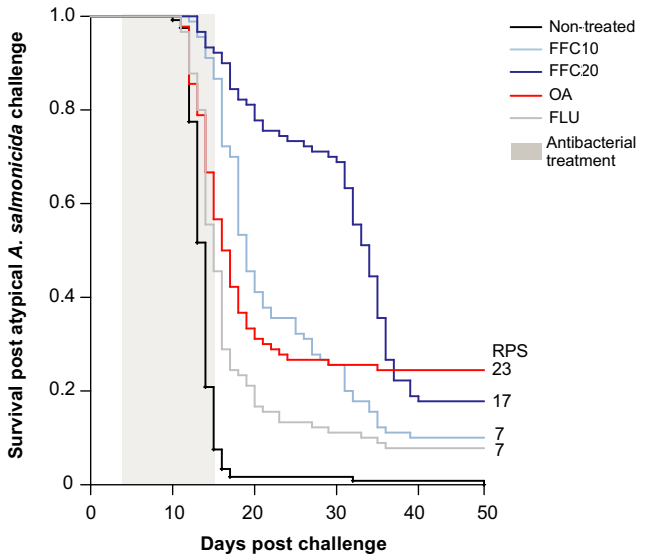

FIGURE 4 Survival curve of lumpfish experimentally challenged with atypical *Aeromonas salmonicida* after antibacterial treatments. Black line is average of non-treated control ($n = 4$ tanks, each with 30 fish in each), light blue line is treatments with FFC 10 mg kg^{-1} day^{-1} for 10 days, dark blue line is treatments with FFC 20 mg kg^{-1} day^{-1} for 10 days, red line is treatments with OA FFC 25 mg kg^{-1} day^{-1} for 6 days, and grey line is treatments with FLU 25 mg kg^{-1} day^{-1} for 6 days (see Table 1 for more details of feeding during the treatment period). Average of three parallel tanks (with 30 fish in each) is shown for all treatment groups. The treatment period is shaded grey. RPS (relative percentage survival) is given for each treatment

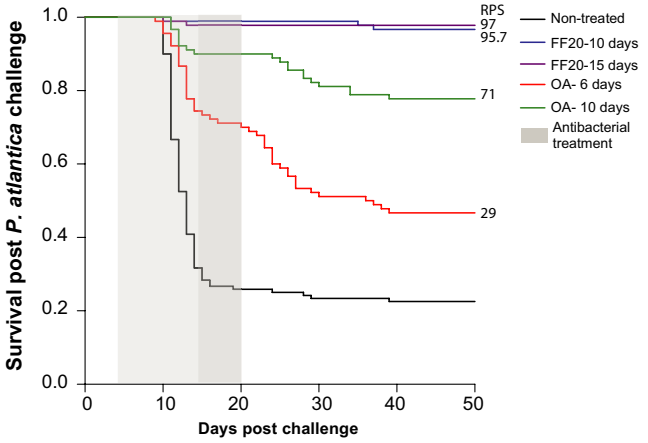

FIGURE 6 Survival curve of lumpfish experimentally challenged with *Pasteurella atlantica* after antibacterial treatments. Black line is average of non-treated control ($n = 4$ tanks, with 30 fish in each tank), light blue line is treatments with FFC 20 mg kg^{-1} day^{-1} for 10 days, dark blue line is treatments with FFC 20 mg kg^{-1} day^{-1} for 15 days, red line is treatments with OA FFC 25 mg kg^{-1} day^{-1} for 6 days, and green line is treatments with OA 25 mg kg^{-1} day^{-1} for 10 days (see Table 2 for more details of feeding during the treatment period). Average of three parallel tanks (with 30 fish in each) is shown for all treatment groups. The treatment period is shaded grey. Statistical analyses are shown in Table S3. RPS (relative percentage survival) is given for each treatment

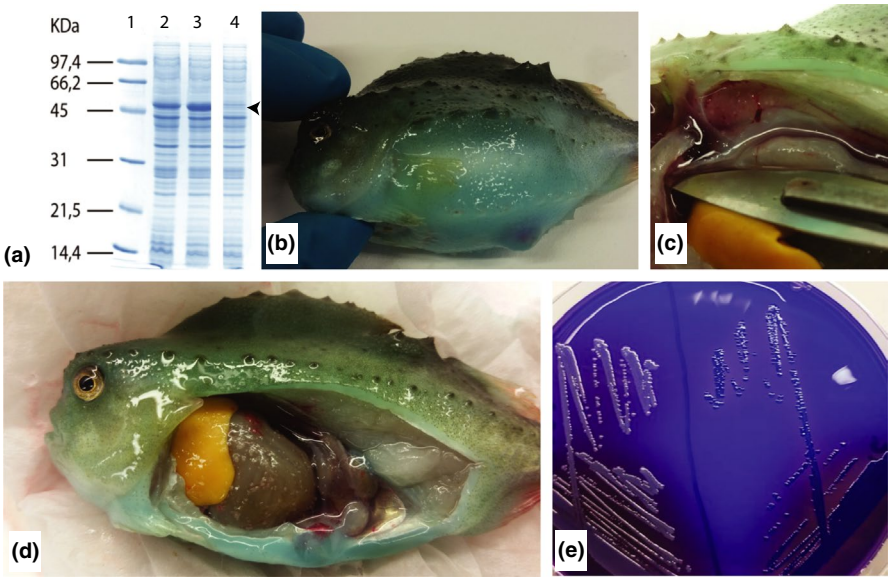

FIGURE 5 Protein profile and macroscopic observation after challenge with atypical *Aeromonas salmonicida*. (a) 12% SDS-PAGE. Lane 1: SDS-PAGE standard, low range molecular markers; Lane 2: start culture grown at 18°C; Lane 3: main culture used for challenge grown at 18°C; and Lane 4: culture grown at 20°C. Arrow indicates A-layer protein, (b) swollen anus, (c) white nodules in the head kidney, (d), white nodules on liver and other organs and (e) reisolated bacteria streaked on the right-hand side of a blue agar plate. A white colony (atypical *A. sal* without A-layer) is shown for comparison

Macroscopic observations of moribund fish were white spots around the eyes and on the skin (Figure 7a), erosion of the lower jaw (Figure 7b) and subcutaneous bleeding on the gill operculum (Figure 7c). Bacteria samples from head kidney were streaked into blood agar plates. Single colonies were further streaked on new plates. Tiny colonies characteristic of *Pasteurella* atlantica were observed after about 4–5 days (Figure 7d).

It is well known that diseased fish often have reduced appetite. This is also true for heavily infected lumpfish, but in this study, lumpfish showed generally good appetite and feed pellets were often

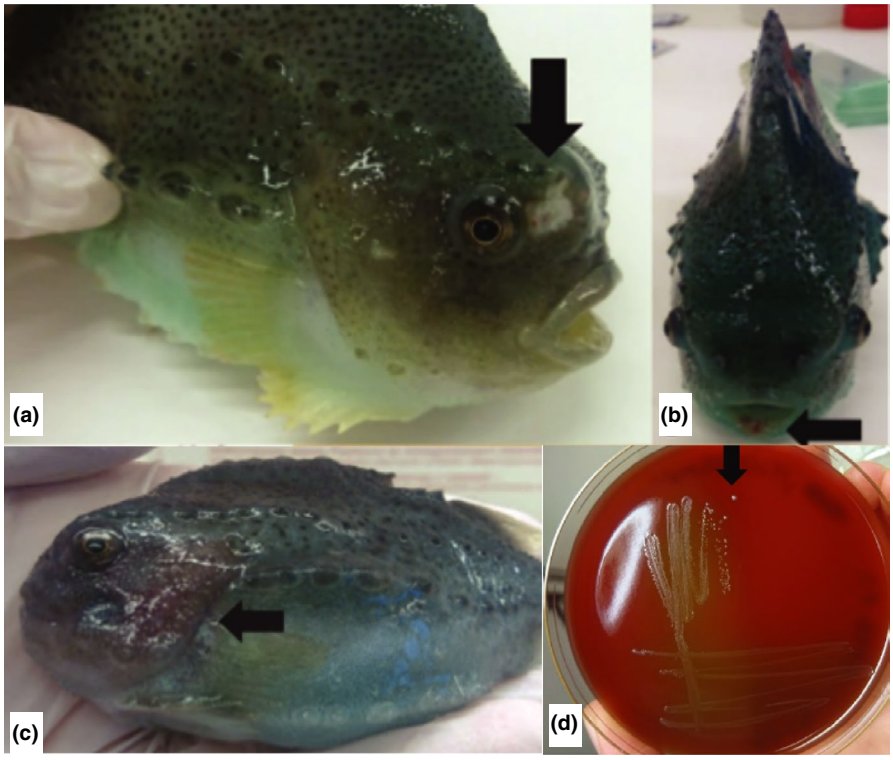

FIGURE 7 Macroscopic observations after challenge with *Pasteurella atlantica*. (a) White spots around eye and on the skin. (b) Erosion of the lower jaw. (c) Subcutaneous bleeding on the gill operculum. (d) Single colony of *P. atlantica* isolated from diseased fish streaked onto a blood agar plate

found in the stomach of moribund and dead lumpfish. Oral administration of the antibacterials is therefore considered a good alternative for lumpfish.

4 | DISCUSSION

Lumpfish are vulnerable to bacterial infections, and antibacterial treatment of diseased fish is needed to maintain good fish welfare. Although medicated feeds are commercially available for lumpfish in Norway, no protocols for recommended doses and treatment regimens are accessible. Therefore, this study was initiated to investigate the efficacy of three antibacterial agents, FFC, OA and FLU, to treat infections caused by *V. anguillarum*, atypical *A. salmonicida* and *P. atlantica* in lumpfish. The three antibacterials were chosen due to availability as medicated feed (FFC and OA) and based on pharmacokinetic properties and increased sensitivity towards FLU compared with OA of some isolates of *A. salmonicida*. FLU was included in this study as an alternative to OA.

In this study, the final cumulative mortalities in the challenged unmedicated groups were 69%, 99% and 77%, respectively, for *V. anguillarum*, atypical *A. salmonicida* and *P. atlantica*. These values are within or close to the recommended mortality range of 50% to 90% (Amend, 1981) and 30% to 70% (Elston et al., 1995) for unmedicated controls suggested for dose titration trials when testing the efficacy of antibacterials in fish. Mortality of these magnitudes has previously been reported in cod when infected with *V. anguillarum* with 82% and 87.5% (Samuelsen & Bergh, 2004; Vik-Mo et al., 2005) and 92% when Atlantic salmon was infected with *A. salmonicida* (Samuelsen et al., 1999). This confirms the validity of the challenge models used in this study.

The high efficacy of the antibacterials and doses tested for the treatment of vibriosis in lumpfish is reflected in the high RPS values ranging from 84% to 98.5%. In previous investigations, these antibacterials have also shown high efficacy in the treatment of vibriosis in other fish species such as halibut (Samuelsen, 1997) and cod (Samuelsen & Berg, 2004; Vik-Mo et al., 2005). Using a similar treatment regimen as in this study, the efficacy of florfenicol and oxolinic acid in the treatment of vibriosis in cod gave RPS values of 61% and 68% (OA) and 64% and 77% (FFC) using doses of 10 and 20 mg/kg body weight d^{-1}, respectively (Samuelsen & Bergh, 2004), whereas Vik-Mo et al. (2005) found an RPS value of 72% for FLU using a daily dose of 25 mg/kg.

The efficacy of antibacterials to treat lumpfish infected with atypical *A. salmonicida* was examined applying the same treatment regimens as described for vibriosis. In this case, the RPS values ranged from 7% to 23%, indicating that none of the tested antibacterials, doses or treatment regimen efficiently treated the infection. It has, however, not established any guidelines that indicate when an RPS value is too low. This was an unforeseen result since the bacterial isolate used for challenge had MIC values that combined with pharmacokinetic values gave PK/PD indices that indicated therapeutic success using OA, FLU and the highest dose of FFC (Kverme et al., 2019; Haugland et al., 2019). Furthermore, these antibacterials have shown high efficacy in treating furunculosis in other marine species such as Atlantic salmon (Michel et al., 1980; Austin et al., 1983; Nordmo et al., 1998; Samuelsen et al., 1999), halibut (Samuelsen, 1997) and goldsinny wrasse (Samuelsen et al., 2002). The doses and treatment regimen used for FLU and OA in this study are identical to those recommended for treating furunculosis in Atlantic salmon. However, since the efficacy for both drugs was poor, further research is needed to determine potential effective

doses and dosage regimens. One option may be to treat lumpfish with OA every day instead of days 1, 2, 4, 6, 8 and 10, as we have seen that OA is excreted rapidly from plasma and tissues of lumpfish (Haugland et al., 2019), and such strategy was more efficient in lumpfish infected with *P. atlantica* (Figure 6). For treatment with FFC, we used the recommended procedure from the producer, as well as double dose (FFC20 mg/kg/day). The groups treated with FFC20 mg/kg/day gave lower mortality than the other groups until 2 weeks after the antibacterial treatment, when a rapid increase in mortality occurred, from 31% to 77% in the next 10 days. It is therefore tempting to suggest that a prolonged period of medication, from 10 to 15 or 20 days, and/or a higher dose could have been sufficient to obtain a satisfactory survival rate. This should, however, be verified experimentally. One major difference between *V. anguillarum* and atypical *A. salmonicida* is that the latter induce formation of white nodules in kidney and other organs in lumpfish (this study and Rønneseth, Brudal, et al., 2017). Although FFC is efficient against intracellular microorganisms such as *Piscirickettsia salmonis* (Henriquez et al., 2016; San Martín et al., 2019), theoretically the nodule structure may reduce access of the antibacterials into the core of the nodule and result in concentrations of antibacterial agents being too low to effectively combat the bacteria. Even a small number of surviving bacteria can be enough to resume the disease. Furthermore, suboptimal concentrations may promote the development of bacteria resistant to antibacterial agents and should be avoided.

As *P. atlantica* has been suggested to be a facultative, intracellular bacterium (Ellul, Bulla, et al., 2019), we hypothesized similar difficulties in combatting this disease as we experienced for atypical *A. salmonicida*. Thus, the dosage regimens were changed to FFC 20 mg kg^{-1} day^{-1} successively for 10 and 15 days, and OA 25 mg/kg administered at days 1, 2, 4, 6, 8 and 10 and 25 mg/kg administered daily for 10 days. High efficacy was found for both dosage regimens using FFC giving RPS values of 95.7% for the 10-day medication regimen and 97% when treated for 15 days. Due to the small difference in efficacy between the two, the suggested dosage regime is therefore 20 mg/kg daily for 10 days. When treating fish with antibacterial agents, it is advantageous to switch between substances with different mechanisms of action to prevent development of resistant bacteria. A major difference in RPS was found using OA where daily administration of 25 mg/kg for 10 consecutive days (total dose 250 mg/kg) gave an RPS value of 71%, whereas 6 administrations over a 10-day period (total dose 150 mg/kg) were much less efficient with an RPS value of 29.1%. Therefore, if OA is to be used as an alternative to FFC in treating pasteurellosis, the suggested dose and dosage regimen is 25 mg/kg daily for 10 days.

This study shows a good relationship between in vitro MIC and the efficacy of the treatment regimen for vibriosis. However, there was no relationship between in vitro MIC and efficacy in treating atypical furunculosis. This is especially evident not only for OA and FLU but also for the highest dose of FFC where PK/PD indices indicated therapeutic success. This shows the importance of performing efficacy studies.

5 | CONCLUSION

The development of bacteria resistant to antibacterial agents is one of the most serious challenges in human and veterinary medicine (Giraud et al., 2006; Cabello et al., 2016; Carvalho & Santos 2016) and highlights the importance of developing treatment regimens based on knowledge.

In the current study, we found that to treat lumpfish infected with *V. anguillarum* it is possible to choose between different antibacterial agents. To treat pasteurellosis, caused by *P. atlantica*, FFC20 mg/kg daily for 10 days is suitable and OA25 mg/kg daily for at least 10 days is an alternative to avoid using the same drug repeatedly. Under the condition tested in the present study, none of the antibacterials were efficient in treating atypical furunculosis in lumpfish.

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CONFLICT OF INTEREST

The authors state that there is no conflict of interests to declare regarding the presented work.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available within the article and its supplementary materials.

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