Received: 2 February 2021 | Accepted: 7 April 2021
DOI: 10.1111/jfd.13381

RESEARCH ARTICLE

Pasteurella spp. Infections in Atlantic salmon and lumpsucker

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Funding information
This work was supported by Institute of Marine Research, internal project number 15196, internal funding by PHARMAQ Analytiq and by the University of Bergen.

Abstract

The use of cleaner fish as a delousing method in Norwegian salmonid aquaculture has increased tremendously over the last few years. This has led to the emergence of a new large industry of farming lumpsuckers (*Cyclopterus lumpus* L.).

The use of lumpsuckers as cleaner fish has, however, not been problem-free. Bacterial diseases cause high mortalities with pasteurellosis as one of the major emerging diseases. During the past few years, outbreaks of pasteurellosis in farmed Atlantic salmon (*Salmo salar* L.) have become more frequent. This has led to an increasing concern that this disease will become common in salmon farming as well. The purpose of this study was to investigate the susceptibility of Atlantic salmon to *Pasteurella* spp. infection and the possibility of lumpsuckers transmitting pasteurellosis to Atlantic salmon. Atlantic salmon were experimentally challenged, either by bath or by cohabitation with challenged lumpsuckers, using two different strains of *Pasteurella* spp. (originating from lumpsucker and Atlantic salmon, respectively). No clinical signs of pasteurellosis were observed on any of the Atlantic salmon. The lumpsuckers were, however, equally susceptible to both isolates. In addition, clear differences in histopathological changes were observed between individuals challenged with the two isolates.

KEYWORDS
bath-challenge, cleaner fish, *Cyclopterus lumpus L.*, histopathology, pasteurellosis, *Salmo salar L.*

1 | INTRODUCTION

The rapid growth of salmonid aquaculture around the world, including Norway, has resulted in an increased pathogen burden. Sea lice (*Lepeoptheirus salmonis* and *Caligus* spp.) are major pathogens affecting the global salmonid industry and the yearly financial loss caused to farmers globally could run as high as €880 million (https://www.intrafish.com/aquaculture/business-intelligence-the-salmon-farming-industrys-biggest-problem/2-1-551557). Frequent use of the few chemotherapeutics available has over the past decade resulted in the development of resistant lice. Thus, new solutions such as biological delousing using cleaner fish have been implemented to control the lice numbers in salmon farms. In Norway and to some extent in the UK and Iceland, lumpsucker (*Cyclopterus lumpus* L.) and various species of wrasse (*Labridae*) are commonly used as cleaner fish. In 2019, approximately 60 million cleaner fish were used for this purpose in Norway alone (statistics from the Norwegian directorates of fisheries). Lumpsuckers are more favoured by the Norwegian farming industry as they are more easily farmed/cultivated compared with wrasse and, also, due to their better delousing activity at low temperatures (Espeland et al. 2010). This has resulted in high demand for this species, with approximately 42 million farmed lumpsuckers,

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being used in 2019 (statistics from the Norwegian directorates of fisheries). As they are a relatively new species introduced to farming, several bacterial diseases have been documented, with the most frequent pathogens including *Vibrio* species, atypical *Aeromonas salmonicida*, *Pseudomonas anguilliseptica*, *Tenacibaculum* spp., *Moritella viscosa* and an unnamed *Pasteurella* sp. among others (Alarcón et al., 2016; Bornø et al. 2016; Marcos-López et al., 2013).

The *Pasteurella* bacterium was first detected in Norwegian aquaculture in Atlantic salmon (*Salmo salar* L.) in the late 1980s and the first detection in lumpsucker came in 2012. Since then, the outbreaks in farmed lumpsuckers have steadily increased. A shift came in the spring of 2018 when incidents of Atlantic salmon suffering from pasteurellosis increased in frequency in certain regions of Norway. Currently, *Pasteurella* bacteria affect lumpsuckers in all life stages, from hatcheries to fish deployed in salmon cages (Alarcón et al., 2016). As *Pasteurella* sp. has been detected in lumpsucker eggs and milt, it is suggested that vertical transmission is possible (Kui, 2017). There are currently no approved commercial prophylactic treatments, including vaccines, available against pasteurellosis in lumpsucker. In addition, studies indicate that lumpsucker surviving pasteurellosis can become asymptomatic carriers of *Pasteurella* sp. (Ellul et al., 2018), meaning re-emergence of the disease cannot be excluded. Signs in diseased lumpsucker may vary according to the intensity of infection and include characteristic white spots over the skin and eyes, frayed fins and haemorrhage at the base of the jaw and fins in the more chronic cases (Ellul et al., 2018,2019).

The *Pasteurella* sp. isolates recovered from diseased lumpsucker in Norway are genetically related to, but serologically distinct from *Pasteurella skyensis* which has been documented in Scottish Atlantic salmon (Alarcón et al., 2016). It is also genetically distinct from although phenotypically similar, to a group of *Pasteurella* isolates recorded to cause a systemic infection characterized by severe ophthalmitis known as "varracalbmi" in Norwegian Atlantic salmon (Valheim et al., 2000). Recently, the presence of *P. skyensis* was confirmed in Atlantic salmon in Norway by the Norwegian Veterinary Institute, 2020, using whole-genome sequencing. With this recent confirmation of *P. skyensis*, there is a growing concern with regard to the negative impact of pasteurellosis infections on the salmon industry.

It is still unclear whether the outbreaks in recent years with *Pasteurella* spp. are the result of infections from a single genotype or represent multiple occurrences of disease caused by a genetically diverse group of related bacteria. Whole-genome sequencing and phylogenetic analysis of *Pasteurella* sp. strains isolated from lumpsucker and Atlantic salmon in Norway have confirmed that the strains are genetically different from *P. skyensis* as the closest sister group. Intragenomic identity within these *Pasteurella* sp. strains has yet to be resolved.

The aim of this study was to challenge Atlantic salmon and lumpsucker with *Pasteurella* isolates originating from both diseased lumpsucker and Atlantic salmon, to investigate the course of the disease and possible horizontal transmission from lumpsucker to Atlantic salmon.

2 | MATERIALS AND METHODS

The trial was approved by the Norwegian Food Safety Authority, National Assignments Department, under approval number: FOTS ID 21,684.

2.1 | Fish and rearing conditions

The experiment was performed in the research facilities at the Institute of Marine Research (IMR) in Bergen. This research facility is approved by the Norwegian food safety authorities as an experimental animal facility and by the Norwegian Veterinary Institute for conducting experiments with pathogens regarded as category 1 (exotic), 2 (non-exotic) and 3 (national) diseases on the Norwegian disease list. It has a flow-through water system, and the inlet water is pumped from a depth of 128 m into sedimentation tanks. There was no UV treatment of the inlet water. The water is heated to the desired temperature using a heat exchange system.

The Atlantic salmon (*S. salar*) used in this experiment was provided by The Aquatic and Industrial Laboratory, ILAB (Bergen). Fish were brought to the IMR's research facility 6 weeks before the start-up of the experiment and smoltified according to standard protocols provided by ILAB. The acclimatization time between finalized smoltification and the initiation of the challenge experiment was a minimum of 3 weeks.

Lumpsuckers (*C. lumpus*) was provided by IMR's research station in Austevoll and was transported to IMR's research facilities in Bergen. The lumpsuckers were acclimatized for 3 weeks prior to the start of the experiment.

To ensure a minimum of handling, both lumpsuckers and Atlantic salmon, with exceptions of the Atlantic salmon included in the cohabitation tanks, were transported to their respective 250 L experimental tanks when arriving at the research facility at IMR. The Atlantic salmon used in the cohabitation tanks were housed in 400 L tanks prior to the start of the experiment.

During the experiment, water flow in all tanks were kept at 500 L/hr and fish were held at a photoperiod of 12L:12D. Water temperature was set to 12°C ± 0.5°C with a salinity of 34.2 ppt.

All fish were fed according to appetite. The Atlantic salmon were fed Spirit trout 3.0 mm (Skretting). Average weight at the start of the experiment was 79 g ± 14 g. The lumpsuckers were fed Otohime C1 at the start of the experiment and changed to Otohime S2 as the fish grew (Pacific Trading Aqua LTD, Ireland). Average weight at the start of the experiment was 30 g ± 6 g.

Both the Atlantic salmon and lumpsuckers were examined by authorized fish health personnel prior to transportation to IMR. No indications of disease were observed, and the fish was declared healthy and provided with a health certificate. In addition, the lumpsuckers were submitted for screening for the *Cyclopterus lumpus Toti-like virus* (CluTLV), *Cyclopterus lumpus corona virus* (CluCV) and *Lumpfish flavi virus* (LFV) at the diagnostics laboratory at Pharmaq Analytiq (PA) before the start-up of the experiment.

2.2 | Bacterial isolates and preparation of challenge material

The *Pasteurella* sp. strain isolate 1 used in this study (hereby referred to as isolate 1) was recovered from diseased Atlantic salmon from a farm in the southern part of Norway, fall 2019. The *Pasteurella* sp. strain isolate 2 (hereby referred to isolate 2) has been described in previous studies (Alarcón et al., 2016; Ellul et al., 2018,2019). This isolate was originally recovered from diseased lumpsucker from a farm site on the southwest coast of Norway. For the experimental challenge, a second passage of both bacterial strains was used. The bacteria were cultured in tryptic soy broth (TSB) (Becton Dickinson) supplemented with 1.5% NaCl and 10% foetal bovine serum (FBS) (Gibco, Lot no. 2,094,466) at 20°C with shaking (200 rpm). The cultures were harvested in the late exponential growth phase and centrifuged at 2,500 g (Beckman Coulter Allegra X-15R) for 15 min at 4°C; cells were washed once in phosphate-buffered saline (PBS) (Lonza, Lot no. 0,000,715,208) followed by centrifugation and resuspension in PBS. Bacterial cell numbers were measured using a cell counter (CASY Model TT (Innovatis) and CASY worX V1.26) and cell numbers adjusted by dilution in PBS. Two-fold dilutions of challenge material were plated on blood agar (2% NaCl) and colony-forming units (CFU) were counted to confirm bacterial concentration in the suspensions used for the challenge.

2.3 | Bath-challenge

A total of twelve identical 250 L tanks in two separate units were used. All tanks were stocked with a total of 50 fish. Duplicate tanks were used for each experimental treatment, including controls. Both salmon and lumpsuckers were bath-challenged using a challenge dose of 1.0×10^6 bacteria/ml for one hour. Atlantic salmon were kept in their respective tanks during bath challenge. The water levels were decreased to 100 L, water flow was stopped, and the tanks were continuously oxygenated throughout the procedure. The lumpsuckers were transferred to smaller static oxygenated challenge tanks (25 L) and exposed to the same challenge dose. After one hour, the water level and water flow were turned back on in tanks holding salmon and the lumpsuckers were transferred back to their respective tanks. The lumpsuckers were carefully transferred between tanks using a hand net. Unchallenged Atlantic salmon cohabiting with challenged lumpsuckers were transferred to the tanks 24 hr post-challenge. Control groups were treated with PBS and handled similarly as the challenged groups. Tanks 1, 3, 5 and 6 were kept in challenge unit one and tanks 2, 4, 7–12 in challenge unit two. Data from tanks with similar treatments (duplicates) will be combined and hereby referred to as one group, groups 1–6. A description of the experimental set-up, fish groups and treatments is presented in Figure 1.

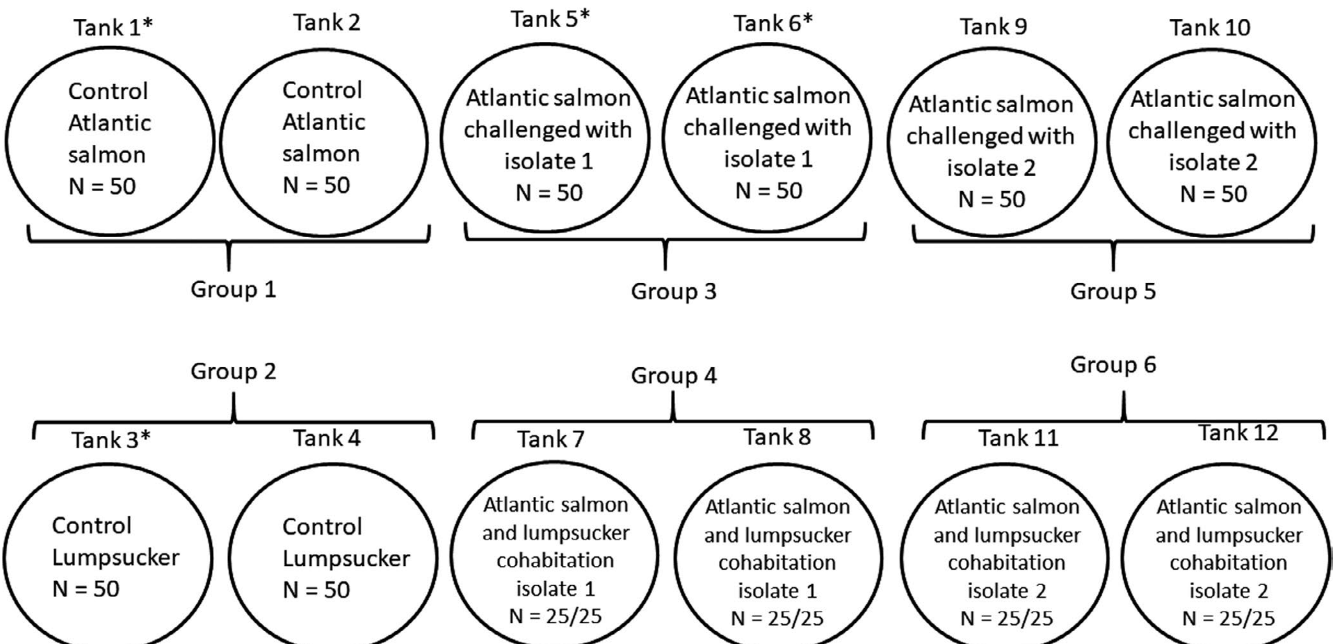

FIGURE 1 Experimental set-up of tanks and treatments. Non-challenged control of Atlantic salmon tanks 1 and 2 = group 1; non-challenged control of lumpsuckers tanks 3 and 4 = group 2; Atlantic salmon bath-challenged with *Pasteurella* sp. isolate 1 tanks 5 and 6 = group 3; cohabiting Atlantic salmon challenged with *Pasteurella* sp. isolate 1 tanks 7 and 8 = group 4; Atlantic salmon bath-challenged with *Pasteurella* sp. isolate 2 tanks 9 and 10 = group 5; cohabiting Atlantic salmon challenged with *Pasteurella* sp. isolate 2 tanks 11 and 12 = group 6; *Tanks located in a separate room

2.4 | Tissue sampling

Sampling was performed weekly, and parallel tissue samples for both PCR analysis and histology were collected. Tissue samples were taken from gills, skin (underneath the dorsal fin and above the lateral line), spleen, heart, kidney and liver. The second left gill arch was sampled for histological analysis, and the third gill arch was sampled for RT-qPCR analysis. Tissue samples used for RT-qPCR analysis were stored in RNA-later at 4°C for 24 hr prior to storage at −20°C or direct RNA extraction. Tissue was sampled from five fish from each of the bath-challenged tanks. Only moribund fish were sampled from the cohabitation tanks.

Tissue samples for histological analysis were fixed in 10% neutral phosphate-buffered formalin (VWR) for approximately 48 hr. The tissue samples were then dehydrated in ethanol, cleared in xylene and embedded in paraffin and stored until further processing.

In addition, bacterial samples were taken from the head kidney and grown on blood agar (BA) with added 2% NaCl (Haukeland University Hospital). Agar plates were incubated at 16°C and screened for bacterial growth every 48 h. Agar plates were kept and screened for bacterial growth for a maximum of 2 weeks. Bacterial colonies were re-cultivated on new BA plates and stored in pure culture in 20% glycerol at −80°C.

One week post-challenge, Atlantic salmon in cohabitation tanks (tanks 7, 8, 11 and 12) started to develop ulcers. In addition to the samples and sampling procedure described above, tissue samples were also collected from the developing ulcers for molecular and histological analysis. In addition, bacterial samples were taken from these ulcers and grown on BA with added 2% NaCl and marine agar (Difco 2,216).

2.5 | Water samples

One litre of water was weekly sampled from each fish tank. The water was immediately filtered through a Thermo Scientific Nalgene Analytical Test Filter Funnels type 0.45 MIC., 250 ml (Thermo Fisher Scientific). The filters were stored at −20°C until further processing by RT-qPCR.

2.6 | Biofilm samples

Samples of biofilm were collected from tanks 5 and 6 at the end of the experiment, using a sterile swab against the tank wall. Water samples from these tanks had previously tested positive for *Pasteurella* sp. isolate 1. The swab was stored in RNA-later at −20°C until further processing by RT-qPCR.

2.7 | RNA extraction and Reverse Transcriptase-quantitative PCR (RT-qPCR)

RNA extraction and RT-qPCR analysis of all samples were submitted for analysis by the commercial diagnostics laboratory at Pharmaq Analytiq (PA) part of Zoetis in Bergen, Norway. Tissue samples were transferred to lysis buffer followed by centrifugation at 6,000 g at 4°C for 10 min. Water filters were soaked in lysis buffer for 15 min and carefully stirred every 2 min. Homogenates were transferred to a Hamilton STAR robot, and total RNA was extracted using MAG-BIND Total RNA kit (Omega Bio-tek), according to the manufacturer`s protocol. Concentration and purity of RNA were determined using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc., Thermo Fisher Scientific).

TABLE 1 Summary of experimental results

Experimental fish group	RT-qPCR detection			Gross pathology		
	Pasteurella spp.	*Tenacibaculum* sp.	*P. perurans*	*Pasteurella* spp.	*Tenacibaculum* sp.	*P. perurans*
Group 1	–	–	–	–	–	–
Group 2	–	–	+	–	–	+
Group 3	–	–	+	–	–	+
Group 4: Atlantic salmon	–	+	+	–	+	+
Group 4: Lumpsuckers	+	+	+	+	–	+
Group 5	–	–	–	–	–	–
Group 6: Atlantic salmon	–	+	+	–	+	+
Group 6: Lumpsuckers	+	+	+	+	–	+

Note: No detection is marked by –, positive detection is marked by +.

[a]*Paramoeba perurans* was detected in the water the last week of the experiment. Group 1: Atlantic salmon control, Group 2: Lumpsuckers control, Group 3: Atlantic salmon challenged with *Pasteurella* sp. isolate 1, Group 4: Atlantic salmon cohabiting with lumpsuckers challenged with *Pasteurella* sp. isolate 1, Group 5: Atlantic salmon challenged with *Pasteurella* sp. isolate 2, Group 6: Atlantic salmon cohabiting with lumpsuckers challenged with *Pasteurella* sp. isolate 2.

RT-qPCR was performed and analysed as described below using elongation factor 1 alpha (ef1-α) from Atlantic salmon or lumpsucker as an internal control. The following specific assays were developed to distinguish between the two *Pasteurella* spp. isolates used in this study (sequences are given in 5`-3` reading frame): *Pasteurella* sp. isolate 1 Fwd: TCTAATATTGATGATCTTGTTTG, Rev: ATTTCCTAAATTAGGAAAGATAC, Probe: ACTTGATGA AGCTACACAACGTG; *Pasteurella* sp. isolate 2 Fwd: TATTAA TGATACAGGTGCTTTA, Rev: ATCCCTTCTGGTTGTGTT, Probe: ACTAATGTTGCAGATCTTGTTTGGC. Eight dilutions (twofold) of RNA were used to create a standard curve and determine the efficiencies of the assays, 0.98 and 1.02 for *Pasteurella* sp. isolate 1 and isolate 2, respectively. All RT-qPCRs were carried out in triplicates. Screening assays were performed on tissues from salmon or lumpsucker simultaneously with the respective ef1-α using qScript XLT 1-step RT-qPCR Toughmix (Quantabio) after the manufacturer`s protocol. Thermal cycling and detection/quantification were performed using Quantstudio™ 5 Real-time PCR system (QuantaBio) under the following conditions: cDNA synthesis; 50°C; 15 min, enzyme activation; 95°C, 2 min, followed by 40 cycles of denaturation of 95°C, 5 s and extension; 55°C; 45 s. Assays used for screening against *Pasteurella* spp. 16S, *Tenacibaculum* spp. and *Paramoeba perurans*, *CluTLV*, *CLuCV and LFV* were developed by PA.

2.8 | Sanger sequencing of bacterial isolates

Bacteria isolated from wounds and kidney were incubated at 15°C for 1–4 days and sub-cultured in 3 × 1,5 ml TSB media supplemented with NaCl and FCS as described above, at 25°C overnight. Total DNA from subcultures was extracted using PureLink™ Genomic DNA Mini kit (Invitrogen) after the manufacturer`s protocol. 16S PCR with 16S Fwd primer: AGAGTTTGATCMTGGCTCAG and 16S Rev primer: CGGTTACCTTGTTACGACTT was set up using qScriptXLT One-Step RT-PCR kit (Quantabio) according to the manufacturer`s recommendations. The following PCR program was used: initial denaturation step 94°C, 2 min and subsequent 35 cycles of amplification (94°C, 30 s; 55°C, 30 s; 72°C, 1.5 min). PCR products were run on a 1% agarose gel for verification of the product. Positive PCR product was cleaned using ExoCleanUp FAST (VWR, Life Science) and sequenced using a Big Dye Terminator v3.1 Cycle sequencing kit (Applied Biosystems®) and analysed in Vector NTI (Life Technologies). *Pasteurella* isolates 1 and 2 were confirmed using isolate-specific RT-qPCR assays (PA). Other bacteria grown and isolated from blood agar plates were further investigated using universal 16S primers. 16S-positive Vibrio bacteria were further typed using *Vibrio* spp. MLST primers (Jolley *et al*. 2018), and 16S-positive *Tenacibaculum* bacteria were further typed by rpoB primers (Småge et al., 2015).

2.9 | Haematoxylin and Eosin (HE), Giemsa and immunostaining

The paraffin-embedded tissue samples were sectioned at 3 µm using a microtome HM 355S with STS. Tissue sections were stained with haematoxylin and eosin (HE) and screened for histopathology and the presence of bacteria.

Giemsa's azur eosin methylene blue solution (Sigma-Aldrich, Cat. No. 109,204) was used to perform the Giemsa staining according to the manufacturer's recommendation for paraffin sections.

Tissue sections were then subjected to immune histochemical staining with a polyclonal antibody (rabbit anti-*Tenacibaculum* sp.)

Histopathological changes			Water/Biofilm			Mortality	
Pasteurella spp.	*Tenacibaculum* sp.	*P. perurans*	*Pasteurella* spp.	*Tenacibaculum* sp.	*P. perurans*[a]	Onset	Final cumulative mortality
–	–	+	–	+	+[a]	Week 2	3%
–	–	–	–	+	+	Week 4	3,5%
–	–	+	+/–	+/–	+[a]/–	Week 10	1%
–	+	+	+	+	+	Week 1	78%
+	–	+	+	+	+	Week 2	90%
–	–	–	+	+	–	Week 5	1%
–	+	+	+	+	+	Week 1	53%
+	–	+	+	+	+	Week 2	>90%

to confirm the presence of *Tenacibaculum* sp. The paraffin sections were incubated at 58°C for 20 min followed by de-waxing in xylene and dehydration with alcohol series and water. Non-specific antibody binding of sections was reduced by incubating with 5% bovine serum albumin (BSA) in TBS for 20 min. All wash steps were carried out with TBS for 5 min until counterstaining. The primary antibody (rabbit anti-*Tenacibaculum* sp.) was diluted 1:400 in TBS containing 2.5% BSA, applied to the sections and incubated at 37°C for 1h, followed by incubation with biotinylated horse anti-rabbit immunoglobulin G and avidin–biotin alkaline phosphatase according to manufacturer's recommendation (Vectastain universal ABC-AP kit; Vector Laboratories). Sections were then stained with ImmPACT Vector Red Substrate kit (Vector Laboratories Inc.). Positive immunostaining showed a red colouration. The slides were then counterstained with Shandon's haematoxylin and covered with a cover glass using mounting media Aquatex (BDH). Screening of tissue sections and photographs were taken using an Olympus BX53 microscope equipped with a Leica DFC 480 Camera and an Olympus U-TV0, 5XC-3 camera adaptor.

3 | RESULTS

A summary of main results is listed in Table 1.

3.1 | Pasteurella infection and re-isolation of Pasteurella spp. from experimentally challenged fish

None of the control fish, neither Atlantic salmon nor lumpsucker, showed clinical signs of a *Pasteurella* infection. All samples tested negative for the presence of *Pasteurella* spp. and the histological examination of tissue samples showed no signs of histopathological changes related to pasteurellosis.

Both *Pasteurella* isolates were re-isolated from kidney and strain typed from lumpsucker from the respective challenged groups, thereby fulfilling Koch`s postulate. We did not manage to re-isolate *Pasteurella* spp. from Atlantic salmon kidney samples.

3.2 | Mortality

No mortality was related to *Pasteurella* infection in the control tanks neither for Atlantic salmon nor lumpsuckers.

The onset of mortality in lumpsuckers challenged with *Pasteurella* sp. isolate 1 (group 4) started 12 days post-challenge (dpc). The mortality persisted for about 1 week, and the final cumulative mortality was about 90% (Figure 2). The remaining fish seemed to attain a more chronic infection where typical white nodules in the kidney could be observed at sampling. Initial mortality observed in lumpsuckers challenged with isolate 2 (group 6) was registered 13 days dpc. Final cumulative mortality and infection status were similar between the two groups challenged with isolate 1 and isolate 2, respectively (Figure 2). Five weeks into the experiment, the remaining lumpsuckers in one of the isolate 1 cohabitation tanks were killed due to reduced health. All Atlantic salmon in the tank had already died or had been sampled. In addition, an increase in mortality was registered in lumpsuckers group two (control) at the end of the experiment most likely caused by an amoebic gill infection.

Even though no mortality related to *Pasteurella* spp. infection was observed for Atlantic salmon, acute mortality was registered for

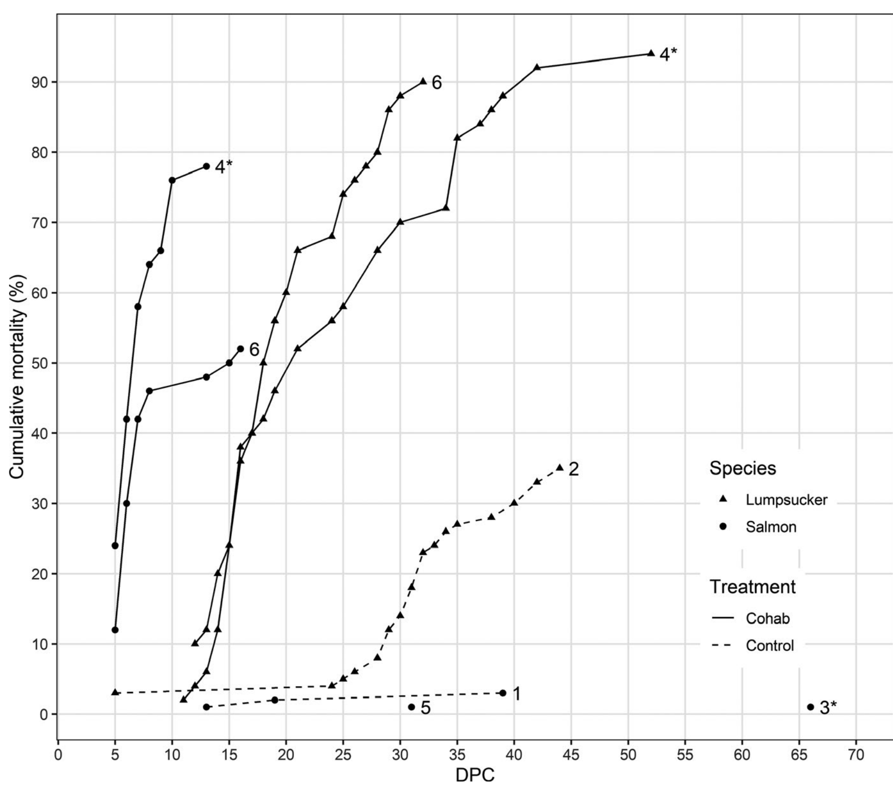

FIGURE 2 Cumulative mortality, days post-challenge (dpc), in respective treatment groups. *Marks groups challenged with *Pasteurella* sp. isolate 1

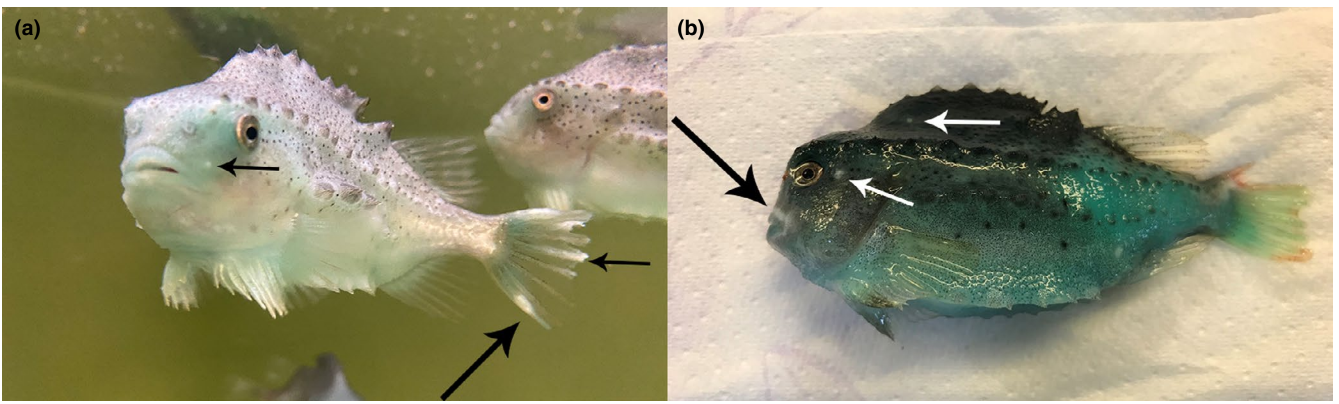

FIGURE 3 (a, b). (a): Lumpsucker challenged with *Pasteurella* sp. isolate 1 showing white spots on fins (marked with black arrow). (b): Lumpsucker challenged with *Pasteurella* sp. isolate 2, showing white spots around the jaws and on the head (marked with black and white arrows), no spots were observed on the fins. Erosion and bleeding on the caudal fin are also seen [Colour figure can be viewed at wileyonlinelibrary.com]

Atlantic salmon cohabiting with lumpsuckers with a *Tenacibaculum* sp. infection. Mortality started about 1 week post-challenge (wpc) and persisted for about 1 week. Cumulative mortality reached between 55% and 80% (Figure 2). Only a few mortalities were registered in groups 1, 3 and 5. In group 1, Atlantic salmon control, three fish died during the experimental period. Only one individual died in bath-challenged Atlantic salmon groups 3 and 5, respectively. No clinical signs of disease were observed during necropsy of these five individuals and none tested positive for neither *Pasteurella* spp., *Tenacibaculum* sp. nor *P. perurans*. Thus, cause of mortality could not be determined.

In group 2, lumpsucker control, mortality started 25 dpc and ended at 44 dpc. A total of 35 fish died.

Mortality and clinical signs related to disease agents other than *Pasteurella* spp. will be further addressed in separate paragraphs.

3.3 | Gross pathology

All lumpsuckers showed clinical signs of a *Pasteurella* infection independent of isolate strain. Typical development of external clinical signs started with white spots appearing on the fins (more notably on the caudal fin), progressing onto the forehead and later appearing on the body of the fish. Fringed fins appeared in the late stages of the disease. White spots and ulcers on the lower jaw and developing ulcers (haemorrhages) on the skin were also seen on some individuals. A few individuals displayed a white circular pattern around the eyes. No change in skin colour was observed. Lumpsuckers challenged with isolate 1 were the first to show clinical signs of pasteurellosis. At 9 dpc, there was a clear difference between the two challenge groups. At this point, lumpsuckers challenged with isolate 1 had developed white spots on both body and fins. The fins were more affected than the body, with the caudal fin and pelvic fins as the most affected (Figure 3a). At the same time point, clinical observations were scarce in the two tanks challenged with isolate 2. In these tanks, signs were delayed by approximately 1 week. Another difference between the two challenge groups was the observation of white spots on the head and mouth. This was more common in lumpsuckers challenged with isolate 2 (Figure 3b).

Internal examination of lumpsuckers revealed classical signs of pasteurellosis with ascites and swollen spleen (Figure 4a) and white

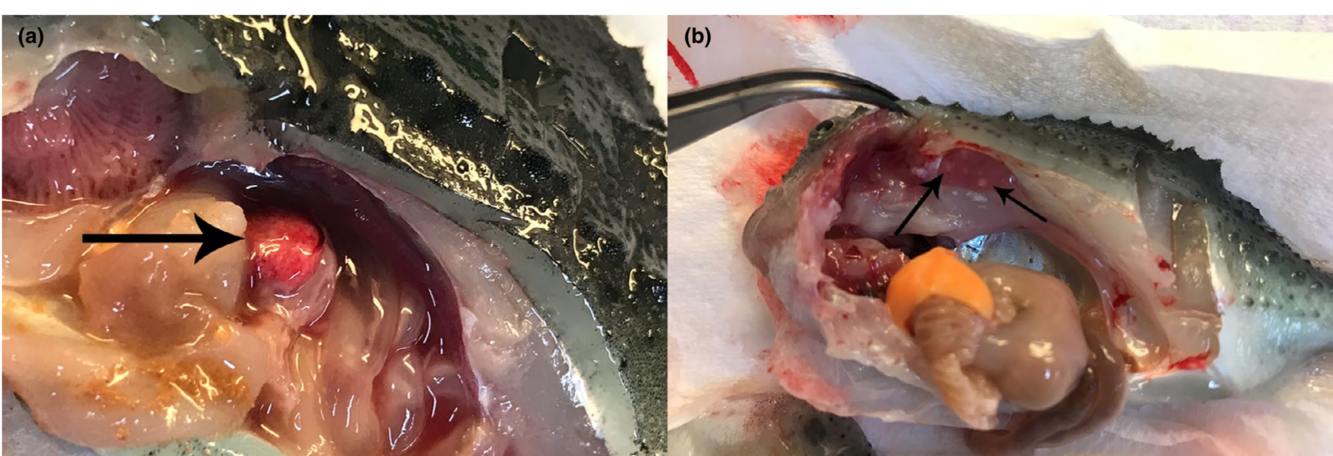

FIGURE 4 (a, b). Lumpsucker challenged with *Pasteurella* sp. isolate 1, sampled 13 days post-challenge. Characteristic white nodules are seen in (a) swollen spleen and (b) kidney (marked with black arrows) [Colour figure can be viewed at wileyonlinelibrary.com]

nodules in the kidney (Figure 4b). Similar pathology was observed for lumpsuckers challenged with isolate 2 (pictures not shown).

No clinical signs of pasteurellosis were observed in any of the bath-challenged or cohabiting Atlantic salmon.

3.4 | PCR results

A total of 206 Atlantic salmon were tested for the presence of *Pasteurella* spp. The fish did not show any clinical signs related to pasteurellosis. Throughout the experiment, only one Atlantic salmon, bath-challenged with isolate 1, tested positive for *Pasteurella* sp. in liver, spleen and kidney tissue with Ct values ranging from 22.1 to 26.2, 2 weeks post-challenge. RNA from the tissue sample of this individual was isolated and analysed twice to confirm the positive test. The only clinical observations were erosion of the pectoral fin on the left-hand side and white spots on the heart. Four individuals, bath-challenged with isolate 2 tested positive for *Pasteurella* sp. in gills and skin tissue (Ct values between 23.6 and 26.7), but no internal organs tested positive in these individuals. All together 109 lumpsuckers including fish from the control group were screened for *Pasteurella* spp. All of the lumpsuckers infected with either *Pasteurella* spp. isolate 1 (n = 25) or isolate 2 (n = 19), tested positive for the pathogen in liver (CT: 11.7–23), spleen (CT: 4.3–16.8), kidney (CT: 6.2–20.1) and heart tissue (CT: 8.1–18.3). Infected lumpsuckers also tested positive for *Pasteurella* isolate 1 or isolate 2 in gills and skin tissue wpc (CT: 10.5–24 4). No individuals in the lumpsucker control group (n = 65) tested positive for any of the *Pasteurella* spp. isolates.

The respective isolates of *Pasteurella* spp. were detected in water samples from all challenge tanks from 1 week post-challenge until three to 1 week before terminating the experiment. The level of *Pasteurella* spp. detected in the water remained stable within each tank. However, small variations were seen between the different tanks with *Pasteurella* sp. isolate 1 generally being detected earlier and in larger quantities compared to isolate 2. No *Pasteurella* sp. was detected in samples collected from group 3 (bath-challenged salmon *Pasteurella* sp. isolate 1).

3.5 | Histopathology

No histopathological changes indicative of pasteurellosis were observed in any of the control groups or groups with challenged Atlantic salmon, including the individual of Atlantic salmon that tested positive for *Pasteurella* sp.

Histopathological examination of tissue samples from moribund lumpsuckers challenged with isolate 2 revealed histopathology consistent with pasteurellosis. Presence of aggregations of small rod-shaped bacteria was observed in the spleen, head kidney, heart and gills (Figure 5a–d, Table 1). Focal necrosis was commonly observed in proximity to the aggregated bacteria but varied between individuals. Haemorrhagic necrosis with infiltration of inflammatory cells in the spleen and kidney was commonly observed, but overall, more extensive in spleen. Bacteria were more evenly distributed throughout the spleen in contrast to the kidney where aggregation of bacteria was in relation to the tubuli. In the heart, bacterial aggregates were observed in the lumen of the atrium (Figure 5d, Table 1).

Histopathological observations of tissue samples taken from lumpsuckers challenged with isolate 1 varied compared with the other challenged group. Overall, the observations of histopathology were scarce among the examined individuals and no clear aggregates of bacteria were observed (Figure 6a–f). However, affected organs showed areas of dissolved necrotic tissue with bleedings and inflammatory cells, and eosinophilic granule cells (EGC) were common. The necrotic areas observed in the various organs appeared less "focal" and more diffuse and spread throughout the whole organ compared with individuals challenged with isolate 2. This was particularly evident when examining the heart (Figure 5c Figure 6e). Histopathological changes and bacteria were only observed in the atrium. Typically, in fish challenged with isolate 1, most of the atrium was affected showing endocarditis, areas of necrosis and inflammation (aggregation of inflammatory cells). However, Giemsa staining of the same tissue samples as stained with HE revealed the presence of bacteria in kidney and atrium of the heart. Bacteria were not observed in dense aggregates but evenly spread and mostly observed in between red blood cells and EGC (Figure 6d,f). There were no observations of bacteria in spleen samples (Figure 6b).

3.6 | Co-infections

3.6.1 | Tenacibaculum sp

Approximately 1 week post-challenge, developing ulcers and mortality of the cohabiting Atlantic salmon could be observed. Mortality was acute and stopped after 3–4 days. Within these few days, approximately 80% of salmon cohabiting in group 4 and 55% in group 6 died (Figure 2). The remaining fish recovered. Bacterial isolates grown from the ulcers of diseased fish were sequenced (16S and rpoB) and the presence of both *Tenacibaculum finnmarkense* and a *Tenacibaculum* sp. was confirmed. Also, immunostaining of skin from salmon with ulcers verified the presence of *Tenacibaculum* sp. (Figure 7).

All fish were screened through the remaining trial period with a general *Tenacibaculum* sp. RT-qPCR assay (PA). A total of 400 gill and skin samples from 200 salmon was tested by RT-qPCR for the presence of *Tenacibaculum* sp. Of these, 16 positive gill samples and three positive skin samples were verified from the Atlantic salmon control group and bath-challenged groups. However, these Atlantic salmon showed no gross pathology related to tenacibaculosis or reduced general health.

Similarly, analysis of a total of 218 gill and skin samples from 109 lumpsuckers resulted in 44 positive gill and 28 positive skin samples.

In addition, *Tenacibaculum* sp. was detected in the water sampled from all tanks throughout the experimental period.

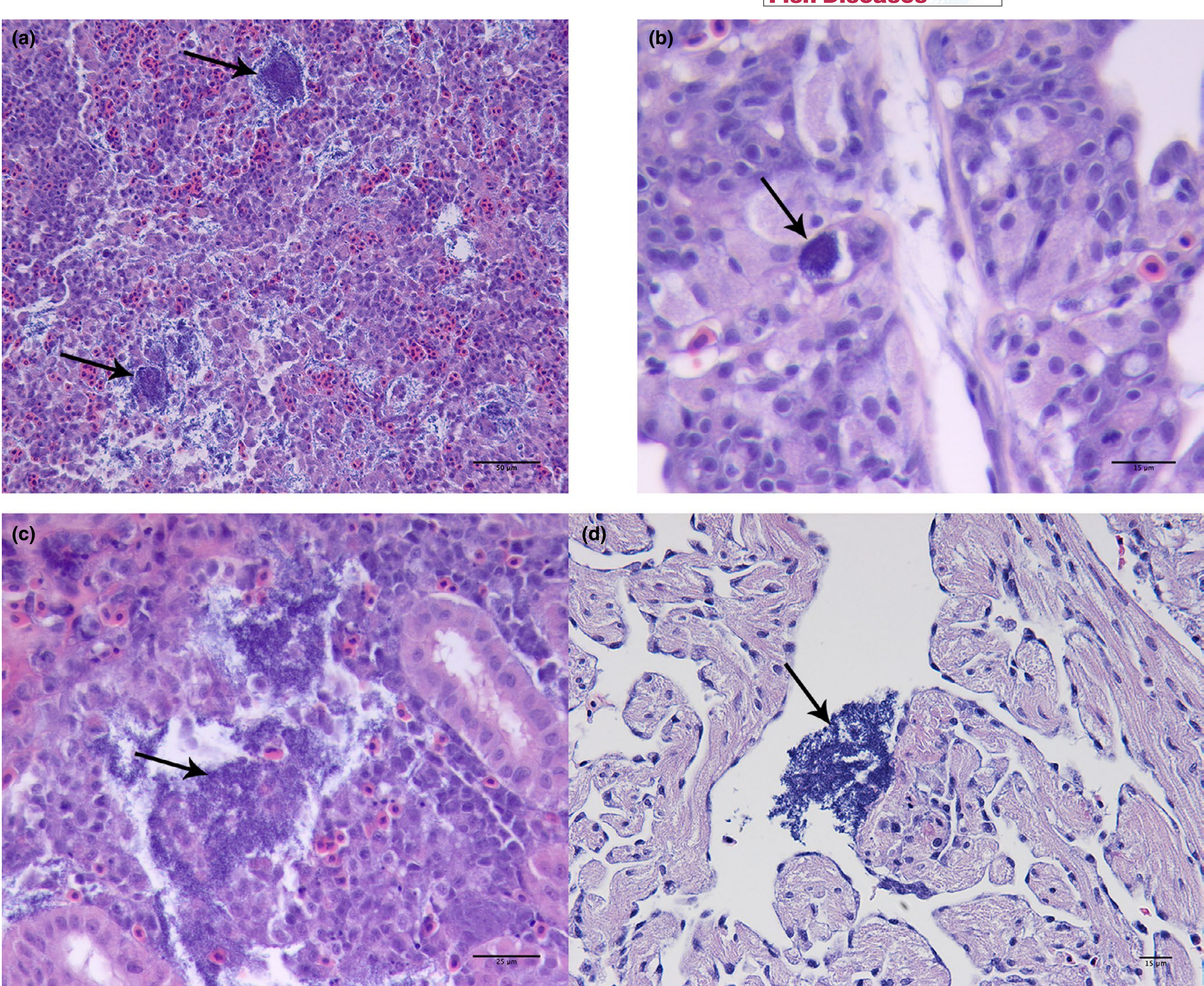

FIGURE 5 (a–d) Lumpsucker sampled 16 days post-challenge (dpc) and 21 dpc, challenged with *Pasteurella* sp. isolate 2, HE staining: (a): Tissue sample of spleen showing multiple aggregates of bacteria (black arrows), magnification 200×, scale bar 50 μm. (b): Aggregation of bacteria in capillary of gills (black arrow), magnification 600×, scale bar 15 μm. (c): Kidney, aggregates of bacteria (black arrow), magnification 400×, scale bar 25 μm. (d): Heart, aggregating bacteria in lumen of atrium (black arrow), magnification 600×, scale bar 15 μm [Colour figure can be viewed at wileyonlinelibrary.com]

3.6.2 | Amoeba

During sampling four wpc, increased mucus production and white/greyish blisters and spots were observed on the lumpsucker gills in both control and challenged groups. At nine wpc, similar clinical signs were observed on the cohabiting salmon. Due to these observations, histological sections and RT-qPCR samples of gill tissue from the start point of the experiment were screened for the presence of amoeba. Histopathological examination of gills from lumpsuckers and cohabiting Atlantic salmon confirmed the presence of amoeba from 2 dpc. The same histopathological changes were observed when examining gill samples from Atlantic salmon from control tank 1 and bath challenge tank 5 at 9 wpc. The amoebic infection was later revealed by RT-qPCR to be caused by *Paramoeba perurans* (Figure 8a,b). Analysis of water samples confirmed the presence of *P. perurans* in all tanks containing lumpsuckers and two tanks (tanks 1 and 5) containing only Atlantic salmon.

4 | DISCUSSION

Outbreaks of pasteurellosis in Norwegian aquaculture have increased over the last few years. Most outbreaks have occurred in lumpsucker, but in recent years, outbreaks on Atlantic salmon have become more common. This has raised the concern of transmission of this bacterium between lumpsucker and Atlantic salmon.

In this experiment, Atlantic salmon were challenged with two different *Pasteurella* spp. isolates by either bath challenge or by cohabitation with lumpsucker suffering from pasteurellosis. None of the Atlantic salmon in this experiment were observed to develop

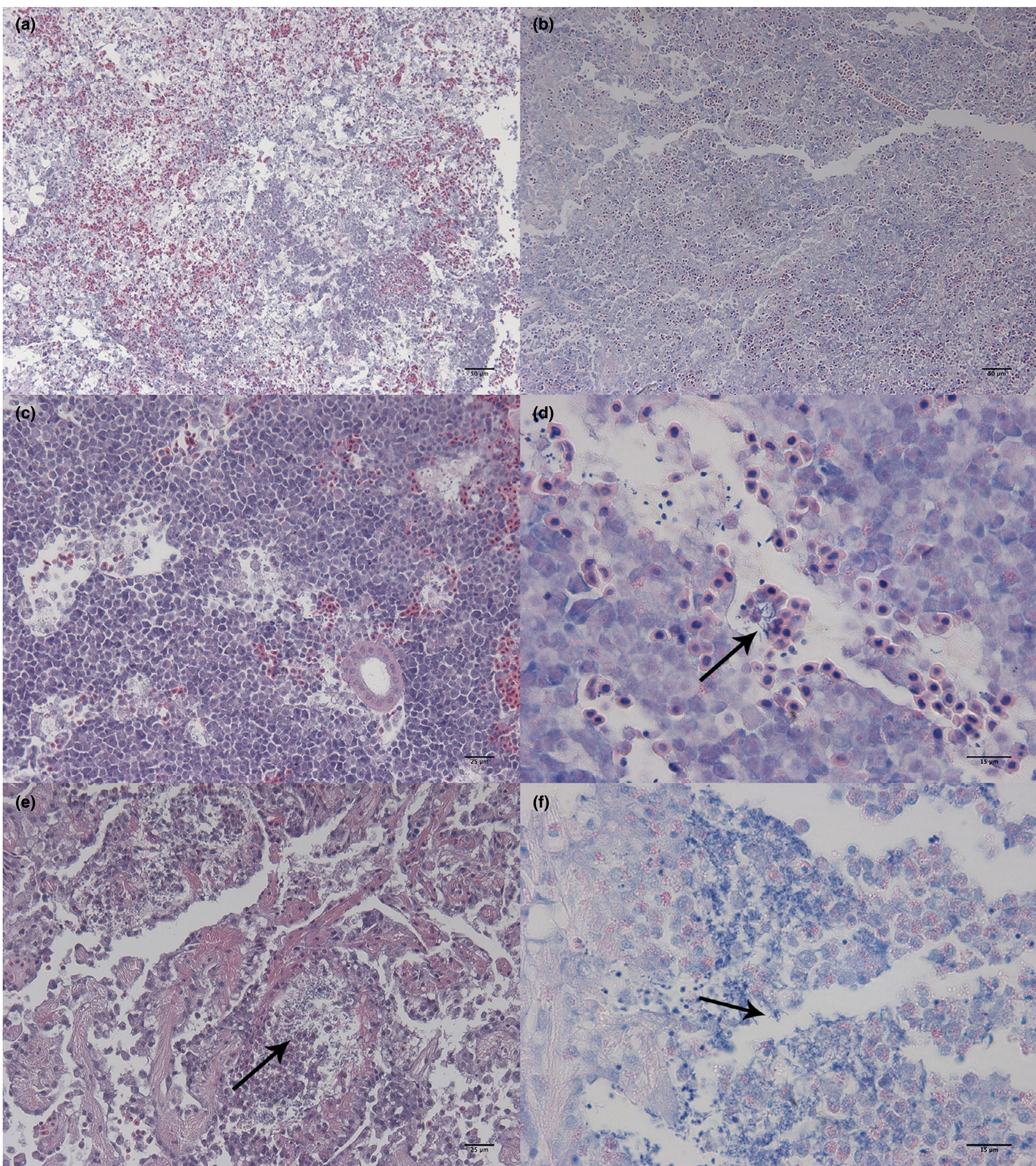

FIGURE 6 (a–f): Lumpsuckers challenged with *Pasteurella* sp. isolate 1, HE staining (a, c, e) and Giemsa staining (b, d, f), respectively. Tissue samples of affected spleen, kidney and heart containing large quantities of EGC in necrotic areas. (a, b): Extensively dissolved spleen with severe bleedings and necrosis. Sample taken 14 days post-challenge (dpc). Magnification 200×, scale bars 50 μm. (c): Kidney sampled 16 dpc, with areas of necrotic dissolved tissue and some bleedings, magnification 400×, scale bar 25 μm. (d): Kidney sample 16 dpc, individual bacteria can be seen between red blood cells (black arrow), magnification 1000×, scale bar 15 μm. (e, f): Atrium of same individual as figure (a, e): Dissolved endocardium and necrotic tissue with inflammatory cells and EGC (black arrow). Magnification 400×, scale bar 25 μm. (f): Individual bacterial can be observed in between EGC (black arrow). Magnification 1000×, scale bar 15 μm [Colour figure can be viewed at wileyonlinelibrary.com]

any clinical signs of pasteurellosis. These findings indicate that Atlantic salmon is less susceptible to pasteurellosis compared with lumpsucker.

Only one Atlantic salmon bath-challenged with *Pasteurella* sp. isolate 1 tested positive in internal organs by PCR analysis. In addition, four Atlantic salmon bath-challenged with isolate 2 tested positive for *Pasteurella* sp. in gill and skin tissue. Although not directly comparable, the CT values indicate an overall lower level of *Pasteurella* sp. in the Atlantic salmon compared with the parallel tissues sampled from lumpsuckers. From this, one can speculate that a higher level of *Pasteurella* bacteria than achieved during this trial needs to be present in Atlantic salmon in order to develop pasteurellosis. Testing of water samples confirmed the presence of both *Pasteurella* spp. isolates in all challenge tanks for up to 4 wpc. Even though we did not quantify the amounts of bacteria present in the water, the level of bacteria remained stable in each tank with only minor variations in CT values between each sampling point. This could indicate that even though the Atlantic salmon did not develop clinical signs of pasteurellosis, the fish shed bacteria into the water, or that the bacteria are present in the system. Some pathogenic strains of the *Pasteurella* family are known to form biofilms in unfavourable environments and may act as a reservoir of infection (Petruzzi et al., 2017). However, the biofilm samples collected from tanks with bath-challenged Atlantic salmon at the termination of the experiment were negative. Biofilm was collected at the edge of the waterline and there is a possibility that *Pasteurella* spp. resides at different locations in the tank even though it is reasonable to believe that the bacteria would have been located in the biofilm at the water edge if present. Swabs from different locations in tanks from land-based facilities have shown that *Flavobacterium* and other bacteria are only present at specific locations in the water system (Testerman et al., 2019). As microorganisms are not evenly distributed throughout the system (Rurangwa & Verdegem, 2015), biofilm should in follow-up experiments, be sampled systematically and regularly at several “hot spots” in the tanks such as inlet–outlet water and areas with lower water circulation throughout the course of the experiment.

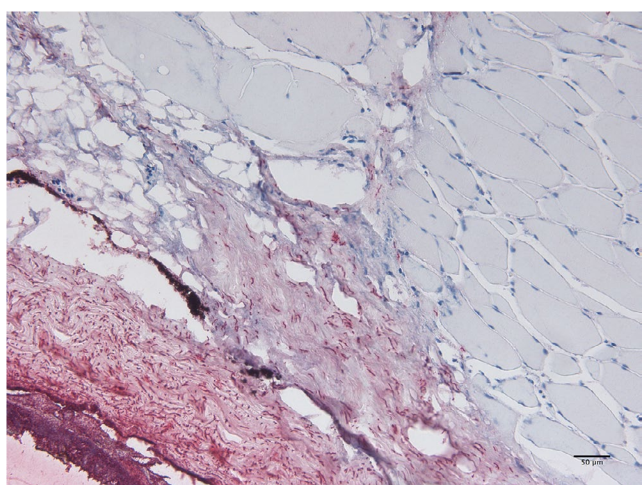

FIGURE 7 Immunostaining, anti-*Tenacibaculum* sp. of Atlantic salmon skin. Sample was taken 6 days post-challenge (dpc). Magnification 200×, scale bar 50 μm [Colour figure can be viewed at wileyonlinelibrary.com]

Another aspect that needs to be considered is the possibility of variable virulence among the *Pasteurella* spp. isolates circulating between and within farm sites causing pasteurellosis in Atlantic salmon. Pasteurellosis is an emerging disease in salmon farming in Norway, and little is known about the strain variations involved in development of disease. Results so far indicate different clusters of *Pasteurella* strains that affect lumpsucker and Atlantic salmon, respectively (Sommerset et al., 2021). But there is currently little knowledge concerning difference in virulence between isolates and the presence and expression of putative virulence factors. The strains used for this experiment were chosen based on previous

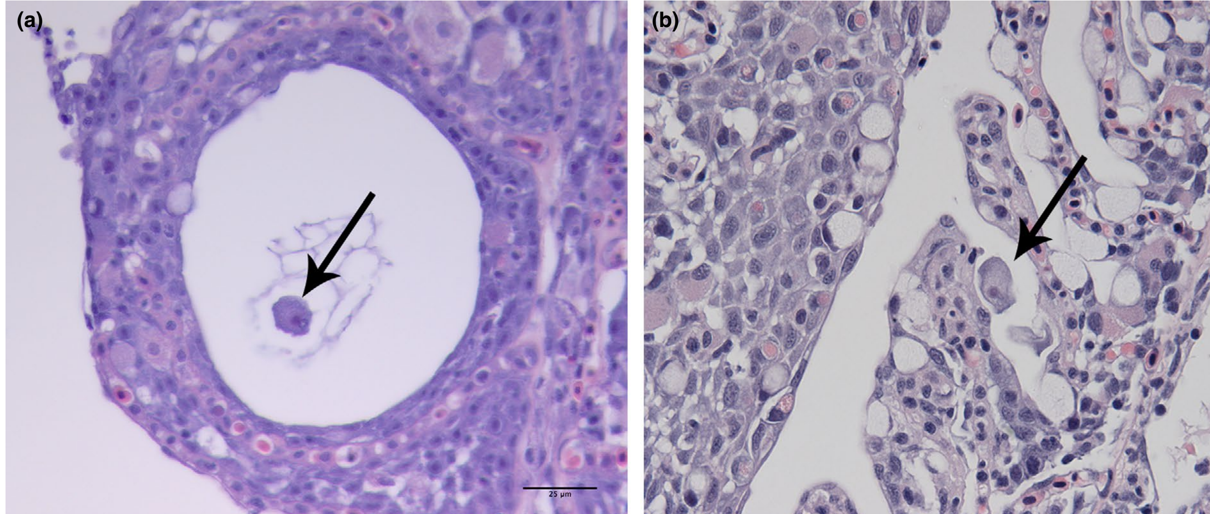

FIGURE 8 (a, b). (a): Amoeba in enclosed lumpsucker gill-lamellas (black arrow), 16 days post-challenge (dpc), magnification 400×, scale bar 25 μm. (b): Atlantic salmon gill sampled 56 dpc with hyperplasia and amoeba between lamellas (black arrow), magnification 600×, scale bar 15 μm [Colour figure can be viewed at wileyonlinelibrary.com]

studies (Alarcón et al., 2016; Ellul et al., 2018, 2019) and *Pasteurella* sp. strains associated with pasteurellosis outbreaks from the field. Differences in intraspecies pathogenicity are well known among many bacterial families. One example is the variations in virulence between different strains of *Tenacibaculum finnmarkense* where the *T. finnmarkense* strain HFJ^T cause higher mortality during outbreaks compared to the *T. finnmarkense* strain Tsp.2 (Olsen et al., 2011). Thus, presence of more virulent strains than the ones used in this present study could explain the lack of development of pasteurellosis seen in Atlantic salmon in this experiment.

Other factors that need to be addressed with regard to the lack of pasteurellosis developing in the Atlantic salmon are the challenge dose used in the bath challenge. The dose used in this experiment is regarded as a medium dose, and it has been proven to be both reproducible and sufficient to cause disease in lumpsucker (Ellul et al., 2018). The aim of this experiment was to determine whether lumpsuckers could transfer *Pasteurella* spp. to Atlantic salmon. Thus, cohabitation groups were designed. The Atlantic salmon bath challenge groups was included to test the susceptibility of Atlantic salmon to the same dosage and strains as lumpsuckers.

The Atlantic salmon cohabiting with lumpsucker suffered a co-infection of both *Tenacibaculum* sp. and later *Paramoeba perurans*. The Atlantic salmon started to develop ulcers, and acute mortality was observed about one wpc. The *Tenacibaculum* sp. infection resulted in a loss of between 55% and 80% of the Atlantic salmon within 2 weeks. This timeframe is most likely too short for development of clinical signs of pasteurellosis and could explain the lack of such in these individuals. However, some fish survived but it is not known whether or not the remaining period of the cohabitation exposure was long enough for the Atlantic salmon to develop pasteurellosis. In the field, most outbreaks of pasteurellosis are mostly related to larger salmon which could suggest that exposure to the bacteria over a longer time period is necessary for an infection to establish itself. *Pasteurella* spp. was detected in the water samples from all challenge tanks containing lumpsuckers, confirming shedding of bacteria into the water. Thus, Atlantic salmon cohabiting with lumpsuckers were, respectively, exposed to both *Pasteurella* isolates. However, the amounts of bacteria shed into the water and the dose the cohabiting salmon is exposed to after initial challenge are unknown. It could be hypothesized that co-infections would make weakened individuals more susceptible to a *Pasteurella* infection. The *Tenacibaculum* infection in the cohabiting tanks should be regarded as simultaneous with the exposure to *Pasteurella* spp. Even though the Atlantic salmon was exposed to a mixture of these pathogens for a period of weeks, *Pasteurella* spp. was not detected in these fish and no clinical signs of pasteurellosis were observed. This brings up again the speculations of the previously mentioned variability in virulence among plausible existing isolates in the field.

The cause of the outbreak of tenacibaculosis in the cohabiting tanks is not clear. Analysis of water samples showed the presence of *Tenacibaculum* sp. in water samples from all tanks, indicating that all fish in this experiment was exposed to this bacterium through the inlet water. However, the additional handling when being transferred to the cohabitation tanks containing the challenged lumpsuckers might have been a contributing factor. Handling as the triggering factor is further substantiated by the fact that no tenacibaculosis was observed in tanks with bath-challenged salmon. No clinical signs of tenacibaculosis, neither gross pathology nor histopathological, were observed in any of the examined lumpsuckers.

In addition to the co-infection with *Tenacibaculum* sp., an amoebic gill infection established itself in all tanks harbouring lumpsucker. The salmon in the cohabitation tanks started developing clinical signs of AGD about 4–5 weeks after the lumpsucker, strongly suggesting horizontal transmission from lumpsucker to salmon. Transmission of *P. perurans* from lumpsuckers to salmon has previously been demonstrated (Haugland et al., 2017). In addition, clinical signs of AGD were observed in tanks 1 and 5, habiting only Atlantic salmon. There are no clear explanations for the AGD developing in the two tanks. Most likely, these two tanks were contaminated due to usage of equipment or splashing of water between tanks. No development of AGD was detected in the remaining tanks.

The fact that farmed Atlantic salmon develop pasteurellosis in a farmed situation suggests that other factors in addition to the *Pasteurella* bacteria have to be present, for an infection to develop. Such factors may be environmental or linked to the general health of the fish such as chronic stress, handling or co-infection with other pathogens. Adding to this impression are reports of pasteurellosis outbreaks typically occurring 2–3 weeks after handling (e.g. sea lice treatment). Much is still unknown when it comes to strain variations and differences in virulence. Systematic work on virulence factors and virulence testing of different strains in vivo will be essential. In outbreaks with *Pasteurella* sp. in Atlantic salmon, other pathogens are more often than not, present, which complicates the "clinical picture" of Atlantic salmon suffering from pasteurellosis. In addition, the farmers have reported that the clinical signs sometimes change from one outbreak of pasteurellosis to the next (personal comments made at Pasteurella webinar 2020). This raises the question of whether or not *Pasteurella* spp. is to be regarded as a secondary pathogen to Atlantic salmon or whether handling and treatment reduce the health of the salmon in a manner that allows for establishment of *Pasteurella* spp. with subsequent outbreaks.

The Atlantic salmon challenged with *Pasteurella* spp. by bath challenge were allowed to acclimate in the experimental tanks with stable conditions prior to challenge and were not exposed to stress factors such as handling or lice treatments. The general health of these experimental fish must therefore be regarded as good.

In contrast to the Atlantic salmon, the lumpsuckers developed pasteurellosis after exposure to both *Pasteurella* spp. isolates in this study. However, a difference in the development of gross clinical signs was observed between the two isolates with the course of disease advancing faster in groups challenged with isolate 1. Furthermore, the location of the typical white spots associated with pasteurellosis appeared different between the two challenge groups. On fish challenged with isolate 1, they appeared on the

head and fins with the caudal fin being most heavily affected. In comparison, on fish challenged with isolate 2, the white spots appeared around the mouth, eye and body (Figure 3a,b). The latter is in accordance with previous challenge studies with lumpsucker isolates (Ellul et al., 2018). In addition, mortality appeared more acute in lumpsucker challenged with isolate 1 compared with isolate 2, which were more gradual. Both isolates caused systemic infection in lumpsucker where white nodules were found in the kidney and spleen, with the spleen being severely swollen in some individuals. Variations in histopathology were observed between lumpsuckers challenged with the two different isolates. Histopathological examination of organs such as kidney, heart, spleen and liver from lumpsuckers challenged with isolate 2 showed bacterial sepsis forming aggregates of bacteria, similar to observations in previous challenge experiments (Alarcón et al., 2016; Ellul et al., 2018).

In contrast, the histopathological examination of tissues sampled from lumpsuckers challenged with isolate 1 did not reveal any distinct bacterial aggregations. However, the observations of severe dissolved, necrotic tissue, inflammation and infiltration of EGC show a severe negative effect on the host caused by this bacterial strain. No systematic comparison of the amount of EGC was performed between control fish and fish from treated groups. In contrast to HE staining, Giemsa staining made it possible to observe bacteria in tissue samples from individuals challenged with isolate 1 (Figure 6d,f). Histopathological observations of chronic pasteurellosis in lumpsucker from field samples are rarely observed. It could be hypothesized that *Pasteurella* isolates of the Atlantic salmon strain do not form aggregates in a similar manner as observed for the lumpsucker strains, making the bacteria difficult to visualize in affected tissue samples using standardized staining procedures such as HE staining. It could also be because most field cases of pasteurellosis seem to be acute, with increased levels of *Pasteurella* spp. in lumpsucker rapidly coinciding with mortality, and without clear histopathological indicators of disease.

The lack of observations of bacterial aggregates may also suggest excretion of exotoxins that damage the tissue. The hypothesis of excretion of exotoxins has previously been suggested by Ellul et al. (2018). It is known that from *Pasteurella multocida* and other bacteria that bacterial SOS system response induced by environmental factors met during host infection can lead to the induction of a lytic cycle causing bacterial lysis and release of exotoxin (Osorio et al., 2015; Pullinger et al., 2003, Balado et al., 2018). However, virulence factors and the possible excretion of exotoxins from Pasteurella isolates originating from lumpsucker and salmon are poorly understood.

5 | CONCLUSIONS

Lumpsucker developed clinical signs associated with pasteurellosis after exposure to both *Pasteurella* spp. isolates tested in the experiment with clinical signs developing more acutely with the Atlantic salmon isolate. This finding indicates lumpsucker to be equally susceptible to *Pasteurella* isolates isolated from both salmon and lumpsucker and that the isolate originating from salmon is highly virulent to lumpsuckers.

The results from the bath challenge, where none of the Atlantic salmon developed pasteurellosis under these experimental conditions, could be an indication that outbreaks of pasteurellosis in salmon farms are the result of an opportunistic infection rather than disease caused by a primary pathogen.

The presence of both *Tenacibaculum* sp. and *P. perurans* during the course of the trial could, in theory, have made Atlantic salmon more susceptible to pasteurellosis, as both a potential stressor and as factors compromising the immune system. However, the mortality caused by the *Tenacibaculum* sp. infection may have altered the infection pressure in the tanks and possibly influenced the results obtained from the cohabitation challenge model.

It is evident that more knowledge on topics such as the difference in isolate virulence, development of disease and evolution of new pathogenic genotypes in polyculture production of fish is essential to increase the welfare of farmed fish.

ACKNOWLEDGEMENTS

The authors would like to thank Åge Høines for guidance and help with preparing figures using R, and Ingrid Uglenes Fiksdal and Dawit Berhe Ghebretnsae for preparing the histological samples. This work was supported by Institute of Marine Research, internal project number 15196, internal funding by PHARMAQ Analytiq and by the University of Bergen.

CONFLICT OF INTEREST

The authors confirm that there are no conflicts of interest.

DATA AVAILABILITY STATEMENT

All data are available from the corresponding author on reasonable request.

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How to cite this article: Sandlund N, Rønneseth A, Ellul RM, Nylund S, Sandlund L. *Pasteurella* spp. Infections in Atlantic salmon and lumpsucker. *J Fish Dis*. 2021;44:1201–1214. https://doi.org/10.1111/jfd.13381