

## Induction of tenacibaculosis in Atlantic salmon smolts using *Tenacibaculum finnmarkense* and the evaluation of a whole cell inactivated vaccine

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### A B S T R A C T

Tenacibaculosis, an ulcerative disease characterised by skin lesions, mouth erosion, frayed fins and tail rot, is a significant fish welfare issue in marine aquaculture worldwide. The disease is a concern to the Atlantic salmon farming industry due to mortality losses and the use of antibiotics. The most commonly isolated bacterium from Northern Norwegian salmon farms during tenacibaculosis outbreaks is *Tenacibaculum finnmarkense*; however it has not been shown to be the causative agent. This study investigates the role of *T. finnmarkense* and closely related strains as the possible agent of tenacibaculosis in smolts recently transferred into seawater. The efficacy of whole cell inactivated vaccines using *T. finnmarkense* was also investigated using the developed challenge model. The results show that *T. finnmarkense* strain HFJ<sup>T</sup> and strain Tsp.2 are causative agents of tenacibaculosis as it presents in Northern Norway without the need for any pre-stressor or co-infection, and that different strains vary in pathogenicity. The pathogen does not appear to spread easily from fish to fish and more work is therefore required to determine possible reservoirs and/or vectors. Even though a whole cell inactivated vaccine using *T. finnmarkense* strain HFJ<sup>T</sup> induces an antibody response, this study did not manage to show any protection against tenacibaculosis induced through a bath infection. Future studies need to include determining pathogen-host interactions and identifying possible antigens for vaccine candidates.

### 1. Introduction

Tenacibaculosis is a significant fish welfare issue in many economically important species all over the world (Toranzo et al. 2005; Avendaño-Herrera et al., 2006), and has been reported in all Atlantic salmon, *Salmo salar* farming regions (Kent 1992; Frelief et al. 1994; Handlinger et al. 1997; Ferguson et al. 2010; Barja 2015; Avendaño-Herrera et al. 2016; Frisch et al. 2017; Småge et al. 2017). The disease is a concern to the Atlantic salmon aquaculture industry due to mortality losses and the use of antibiotics (Bornø and Lie 2015). Three species of *Tenacibaculum* (Gram-negative marine bacteria found worldwide) have been associated with this disease in Atlantic salmon: *Tenacibaculum finnmarkense*, *Tenacibaculum dicentrarchi* and *Tenacibaculum maritimum* (Barja 2015; Avendaño-Herrera et al. 2016; Grothusen et al. 2016; Frisch et al. 2017; Karlsen et al. 2017b; Småge et al. 2017; Bridel et al. 2018). Tenacibaculosis is an ulcerative disease characterised by skin lesions, mouth erosion, frayed fins and tail rot (Toranzo et al. 2005).

In Norway, *Tenacibaculum* spp. like bacteria have been identified histologically from skin ulcers since the late 80s (Olsen et al. 2011); however the bacteria were not identified during routine diagnostics due to the use of agars that do not support *Tenacibaculum* growth. The increased use of marine agar, Difco, 2216 (MA) in recent years has resulted in the recovery and identification of *Tenacibaculum* spp. from outbreaks of ulcerative disease (Bornø and Sviland 2011; Takle et al. 2015; Småge et al. 2017). Although *Tenacibaculum* isolates recovered from tenacibaculosis outbreaks in Norway are closely related to *T. finnmarkense* and *T. dicentrarchi* (Habib et al. 2014; Småge et al. 2015; Karlsen et al. 2017b; Olsen et al. 2017; Småge et al. 2017; Bridel et al. 2018), novel species are likely to exist among the recovered isolates due to their large genetic variation found using multilocus sequence analysis (Olsen et al. 2017).

Outbreaks of tenacibaculosis in Northern Norwegian salmon farms has been shown to be dominated by *T. finnmarkense* or closely related strains (Småge et al. 2017) and are most commonly associated with

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mouth erosions and frayed fins (Hjeltnes et al. 2017; Småge et al. 2017). Smolts in land-based saltwater recirculation facilities or ones recently transferred into saltwater net-pens seem to be particularly susceptible (Bornø and Lie 2015; Småge et al. 2017). The susceptibility of salmonid smolts to *Tenacibaculum*-like bacteria has been reported over several decades (Rucker 1959; Borg 1960; Anderson 1969; Sawyer 1976; Kent et al. 1988; Frelier et al. 1994; Olsen et al. 2011; Bornø and Lie 2015; Småge et al. 2017). One possible explanation is the changes in skin microbiota, as well as the physiological changes which occur when smolts are transferred from freshwater to saltwater (Lokesh and Kiron 2016).

Historically, ulcerative disease in Atlantic salmon aquaculture has been associated with the bacterium *Moritella viscosa*, which has been shown to cause disease on its own (Lunder et al. 1995; Bruno et al. 1998). However, recent outbreaks of skin lesions, particularly in warmer temperatures (above 8 °C) have been associated with bacterial infections with *Tenacibaculum* spp. (Småge et al. 2017). These findings are supported by the presence of *Tenacibaculum*-like bacteria in historical histological samples (Olsen et al. 2011). They have been thought to be secondary invaders, only causing disease with previous skin damage (e.g. scarification) or when co-infected with *M. viscosa* (Olsen et al. 2011).

This study investigates the role of *T. finnmarkense* and closely related strains in causing tenacibaculosis in smolts recently transferred into seawater. The efficacy of whole cell inactivated vaccines using *T. finnmarkense* was also investigated.

## 2. Materials and methods

### 2.1. Fish husbandry

All fish experiments presented in this paper were conducted at the Aquatic and Industrial Laboratory (ILAB), Bergen, Norway, using Atlantic salmon smolts provided by ILAB. All fish were confirmed negative by ILAB for the presence of known pathogens of Atlantic salmon (heart screened for *Piscine orthoreovirus* and *Piscine myocarditis virus*; kidney screened for *Infectious pancreatic necrosis virus*; gill screened for *Infectious salmon anaemia virus*). Parent fish were screened and found negative for salmonid alpha virus. The skin was screened prior to transfer to saltwater with real-time RT-PCR and found negative for the presence of *Tenacibaculum* spp., *M. viscosa*, *F. psychrophilum*, and *Yersinia ruckeri*.

For the duration of each experiment, the photoperiod was 12 h (i.e. 12 h light: 12 h dark). Water flow was 300 L per hour per tank and the outlet water in all tanks had a minimum of 77% oxygen saturation. Fish were fed ad libitum with the commercial dry feed Nutra Olympic (Skretting) and were checked a minimum two times a day on weekdays and once a day during weekends. Fish were starved for 48 h prior to any handling (e.g. transfer, vaccination).

All fish showing signs of disease and/or behavioural changes during the experiments were removed from the tanks and euthanized. However, due to the rapidity of the disease, this was not always feasible. For the purpose of this study, the word mortality includes both fish that were euthanized due to animal welfare and fish found dead in the tanks. All fish taken out during or at the termination of the experiments were euthanized with either an overdose of tricaine methanesulphonate, Tricaine PHARMAQ (TMS) or a swift blow to the head. The animal experiments were approved by the National Animal Research Committee (now governed by the Norwegian Food Safety Authority, Mattilsynet) (Experiment ID: 2015/237969, 2015/30245).

### 2.2. Real-time RT-PCR assay development for *Tenacibaculum* spp.

Two different real-time RT-PCR assays were developed during this study due to the lack of published assays for *Tenacibaculum* spp. commonly found in Norway. These were used to screen fish tissues prior to and during the fish experiments. One of the assays targets the

housekeeping (HK) gene, *tuf* (Tb\_tuf: forward AGTGTGACGTCCACCTT, reverse CTGTAAGCCAGGTTCTGT, probe TTTCAATACATACACCTC AGC (Småge et al. 2017)), and the other targets another HK gene, *rpoB* (Tb\_rpoB: forward GGAGCAAACATTGACCAAAT, reverse GGATATGTC CGTAACGTGGAA, probe TCCTGCTTGATCAGTTAAAGCGT). The specificity of both assays was determined by testing each one against RNA extracted from pure cultures of *Vibrio splendidus*, *M. viscosa*, *Flavobacterium* spp., *Aliivibrio* spp., *Tenacibaculum ovolyticum*, *Tenacibaculum soleae*, *T. maritimum*, *T. dicentrarchi*, and *Tenacibaculum* spp. Norwegian field isolates from Småge et al. (2015). After the concentration of primer and probe was optimized, the efficacy of each assay was determined using a six point 10-fold dilution in triplicate of RNA extracted from a pure culture of *T. finnmarkense* strain HFJ<sup>T</sup>. The RNA was extracted following the manufacturer's protocol (5 Prime), except that a second washing step using 100% ethanol was performed prior to air drying of the RNA pellet. The obtained RNA was stored at –80 °C. All assays were run using an AgPath-ID kit (Thermo Scientific) with 2 µL of RNA and the standard AgPath-ID concentrations of primers (400 nM) and probe (120 nM), as these were the optimal concentrations. Each run consisted of 45 cycles.

### 2.3. Challenge material

The *T. finnmarkense* strains used in this study (strain HFJ<sup>T</sup> and strain Tsp.2) were recovered from tenacibaculosis outbreaks in Northern Norwegian Atlantic salmon farms (Småge et al. 2015). The *T. dicentrarchi* strain 35/09<sup>T</sup> used in this study was obtained from NCIMB. It was included as a comparison as it is the most closely related species to *T. finnmarkense* (Bridel et al. 2018), is commonly found in Norway (Småge et al. 2015; Olsen et al. 2017; Småge et al. 2017), and has been shown to cause disease in salmon in Chile (Avenidaño-Herrera et al. 2016). The bacteria were grown on MA at 16 °C for 48 h and stock solutions were stored at –80 °C.

The challenge material was produced by inoculating 1 L of Marine Broth, Difco, 2216 (MB) in 2 L Erlenmeyer flasks with a small amount of frozen bacterial stock solution (400 µL). These were incubated between 60 and 72 h at 16 °C and 140 rpm. Bacterial counts were calculated using the most probable number (MPN) method (Cochran 1950; Blodgett 2010) with 10 fold dilutions in duplicate using 8 replicates per dilution. The average MPN of the duplicate was used as the culture bacterial concentration to calculate the bath concentrations retrospectively. The amount of challenge material required for the target bath concentration was based on previous experiments (data not shown).

### 2.4. Cohabitation experiment

Eight groups of 50 fish with an average weight of 55 g (20 shedders and 30 cohabitants) were used for this experiment (Table 1). The smolts were transferred to 4 °C saltwater (34 ppt) and acclimatized over six weeks to these water conditions. The temperature was chosen based on the seawater temperature during the initial tenacibaculosis outbreak from which *T. finnmarkense* strain HFJ<sup>T</sup> was isolated. Groups 1 and 2 were exposed to *T. finnmarkense* strain HFJ<sup>T</sup>, groups 3 and 4 to *T. finnmarkense* strain Tsp.2, groups 5 and 6 to *T. dicentrarchi* strain 35/09<sup>T</sup>, and groups 7 and 8 were controls, bath exposed with MB (Table 1). Each group pairs were treated as duplicates. Prior to being bath challenged, the shedder fish were anaesthetised with TMS and adipose fin clipped for marking purposes, and then transferred into the 150 L challenge tanks. For the bath infection, the water level in each tank was lowered to a volume of 60 L and flow was shut off. The challenge material was added directly to each tank. After 5 h, water flow was turned back on. The temperature was kept between 4 and 6 °C during this time. Oxygen was provided through diffusors, and oxygen saturation and fish behaviour were monitored every hour. The bath concentrations used for each isolate (Table 1) was based on pilot studies (data not shown).

**Table 1**

Experimental groups for the cohabitation experiment showing isolate and bath bacterial concentrations used. Resulting accumulated mortality is shown. The shedder mortality in groups 1 and 2 are shown in Fig. 1.

Group	Isolate	Amount added (mL) to 60 L bath	Bath concentration (cells mL <sup>-1</sup> )	Number of fish	Accumulated percent mortality	Start of mortality (days post-exposure)	End of mortality (days post-exposure)
1	<i>T. finnmarkense</i> HFJ <sup>T</sup>	50	4.88 × 10 <sup>5</sup>	20 shedders	80	3	10
				30 cohabitants	3.3	25	25
2	<i>T. finnmarkense</i> HFJ <sup>T</sup>	50	4.88 × 10 <sup>5</sup>	20 shedders	80	3	18
				30 cohabitants	6.6	8	8
3	<i>T. finnmarkense</i> Tsp.2	200	9.17 × 10 <sup>5</sup>	20 shedders	10	8	26
				30 cohabitants	16.6	10	35
4	<i>T. finnmarkense</i> Tsp.2	200	9.17 × 10 <sup>5</sup>	20 shedders	15	5	12
				30 cohabitants	20	9	36
5	<i>T. dicentrarchi</i> 35/09 <sup>T</sup>	200	2.42 × 10 <sup>6</sup>	20 shedders	20	5	32
				30 cohabitants	6.6	19	28
6	<i>T. dicentrarchi</i> 35/09 <sup>T</sup>	200	2.42 × 10 <sup>6</sup>	20 shedders	25	3	41
				30 cohabitants	6.6	8	27
7	Control – Marine Broth	200	N/A	20 shedders	15	12	32
				30 cohabitants	6.6	5	5
8	Control – Marine Broth	200	N/A	20 shedders	15	8	19
				30 cohabitants	10	8	27

Cohabitants were added to each tank 24 h after the shedders were challenged. The experiment was terminated after six weeks.

## 2.5. Fish sampling

Samples from the margins of mouth and skin lesions were streaked onto MA. The plates were incubated at 16 °C for a minimum of 72 h. Colonies that matched the morphological characteristics of *Tenacibaculum* (pale yellow pigmented round colonies with long, thin rods shaped cells) were sub-cultured onto MA and incubated for 48 h at 16 °C. A minimum of two cultures per group were cryopreserved at –80 °C. The identification of the recovered bacteria was performed as described in Småge et al. (2015) using the housekeeping gene, *atpD*. The recovered sequences were compared to the challenge material using AlignX in Vector NTI, Invitrogen. Tissues from representative diseased individuals were sampled for histopathology as described in Småge et al. (2016).

To determine the potential presence of bacteria in external and internal tissues of non-diseased cohabitants, samples (size of a match tip) of gills, heart, kidney and skin under the jaw were collected and kept at –20 °C. Five fish per group (including the control groups) were randomly sampled four weeks and six weeks post-transfer into the shedder tanks. Cultured *Halobacterium salinarum* DSM 3754<sup>T</sup> cells suspended in PBS were added to each sample (5 µL) prior to RNA extraction as an exogenous control in the real-time RT-PCR analysis (Andersen et al. 2010). The RNA was extracted as previously described and the obtained RNA was stored at –20 °C.

The extracted RNA was tested for the presence of *Tenacibaculum* spp. using the developed *Tb\_rpoB* real-time RT-PCR assay, an assay targeting the exogenous control (*H. salinarum*) (Andersen et al. 2010), and an assay targeting the elongation factor 1 alpha (EF1A) (Olsvik et al. 2005). All assays were run as previously described. A positive control was included for each run using RNA from skin tissue positive for *T. finnmarkense* that was spiked with *H. salinarum* prior to RNA extraction. Negative controls for both the RNA extraction and the real-time RT-PCR were also included.

## 2.6. Vaccination experiment

Two monovalent oil adjuvanted vaccines were produced: *T. finnmarkense* HFJ<sup>T</sup> with high antigen content (1 ×) and *T. finnmarkense* HFJ<sup>T</sup> with low antigen content (0.06 ×). The vaccine with the high antigen content contained 17 times more bacterin than the vaccine with low antigen content. The bacterin was prepared by growing *T. finnmarkense* HFJ<sup>T</sup> in MB using a 2 L fermentor (1.5 L growth medium

inoculated with 2% pre-culture) for 24 h at 15 °C to a final OD<sub>600</sub> of 5.2. Purity of the bacterial culture was verified by growing the bacteria for an extended period of time on several different suitable agars. The culture was inactivated with 0.4% formaldehyde and concentrated approximately 8 times by centrifugation. It was then formulated into two monovalent oil adjuvanted vaccines (with either 1 × or 0.06 × bacterin concentration) using mineral oil and prepared by a Silverson LR5 rotor-stator mixer according to standard procedures for PHARMAQ vaccines. Sterility of the vaccines was verified by standard procedures.

Two groups of 160 parr of an average weight of 26 g were intraperitoneally (IP) vaccinated with a 0.1 mL dose of one of the two formulated vaccines, and a third group (negative control) of 160 parr were IP vaccinated with 0.1 mL PBS. For this procedure, the fish were anaesthetized using TMS and marked by adipose fin clipping or maxilla trimming to identify each group. The vaccinated fish were mixed and kept in 12 °C freshwater in 500 L tanks. Fish showed no signs of adverse reactions to the vaccine during the immunization period.

After 8 weeks under these conditions, the fish were triggered to smoltify by increasing their photoperiod to 24 h. At 12 weeks post-immunization (approximately 1000 degree days), the smolts were anaesthetized with TMS, sorted into their respective groups (Table 2) and transferred into saltwater in eight 150 L tanks. The salinity was increased gradually over the first 24-h period and the temperature was decreased to 4 °C over the following 2 weeks. Tank conditions were the same as what was used for the cohabitation experiment. The challenge material was produced as described above with the isolates and concentrations described in Table 2.

At 0 (unvaccinated fish), 8 and 12 weeks (approximately 675 and 1000 degree days) post-vaccination, 10 fish were euthanized with an overdose of TMS and blood sampled from the caudal vein. The blood samples were centrifuged and the blood plasma stored at –20 °C for subsequent ELISA analysis.

## 2.7. ELISA

Microtiterplates (Maxisorp™, Nunc) prepared with 5 µg mL<sup>-1</sup> Poly-L-lysine (Sigma) were coated by adding 100 µL of inactivated bacteria, resulting in an OD<sub>600nm</sub> of about 0.05. After washing with PBS containing 0.05% Tween-20 (Merck) (PBST), the plates were blocked at room temperature for 2 h with 5% skimmed milk in PBST. The plasma samples were diluted 2-fold, starting with a dilution of 1:50 in PBST with 1% skimmed milk, and 100 µL was added to each well before incubated at 4 °C overnight. A monoclonal antibody mouse anti-Rainbow Trout Immunoglobulin (cross-reacting with Atlantic salmon IgM-A, produced in-house) (Thuvander et al. 1990) was diluted 1:3500 in PBST

**Table 2**

Experimental groups for the vaccine experiment indicating number of fish per vaccine in each group (A: *T. finnmarkense* strain HFJ<sup>T</sup> (0.06 × /low antigen concentration), B: *T. finnmarkense* strain HFJ<sup>T</sup> (1 × /high antigen concentration), C: Control (PBS)), and showing isolate and bath bacterial concentrations used. Resulting accumulated mortality is shown.

Group	Isolate	Bath concentration (cells mL <sup>-1</sup> )	Vaccine group	Number of fish	Accumulated percent mortality	Start of mortality (days post-exposure)	End of mortality (days post-exposure)
1	<i>T. finnmarkense</i> HFJ <sup>T</sup>	3.5 × 10 <sup>5</sup>	A	20	95	5	9
			B	20	65	5	13
			C	20	90	4	13
2	<i>T. finnmarkense</i> HFJ <sup>T</sup>	3.5 × 10 <sup>5</sup>	A	20	95	4	8
			B	20	90	3	8
			C	20	95	4	9
3	<i>T. finnmarkense</i> HFJ <sup>T</sup>	7.1 × 10 <sup>5</sup>	A	20	100	3	6
			B	20	100	3	7
			C	20	100	3	6
4	<i>T. finnmarkense</i> HFJ <sup>T</sup>	7.1 × 10 <sup>5</sup>	A	21	100	3	6
			B	20	95	3	6
			C	20	100	3	7
5	<i>T. finnmarkense</i> Tsp.2	1.6 × 10 <sup>6</sup>	A	20	45	6	11
			B	20	25	5	10
			C	20	30	4	9
6	<i>T. finnmarkense</i> Tsp.2	1.6 × 10 <sup>6</sup>	A	20	60	6	8
			B	20	50	4	10
			C	20	45	6	12
7	<i>T. finnmarkense</i> Tsp.2	3.3 × 10 <sup>6</sup>	A	20	50	5	10
			B	20	40	4	9
			C	20	35	5	9
8	<i>T. finnmarkense</i> Tsp.2	3.3 × 10 <sup>6</sup>	A	19	84	4	8
			B	20	75	3	8
			C	20	65	3	8

with 1% skimmed milk. 100 µL was added to each well and the plates were incubated one hour at room temperature. The secondary antibody rabbit anti-Mouse Immunoglobulin conjugated to alkaline phosphatase (Dako) was diluted 1:500 in PBST with 1% skimmed milk, and 100 µL was added to each well followed by one hour incubation at room temperature. The plates were washed three times with PBST between the incubations. Bound antibodies were detected by adding 100 µL substrate *p*-nitrophenyl-phosphate (Sigma) in 10% diethanolamine buffer, pH 9.8 (Sigma-Aldrich) to each well and the colour reaction was read at OD<sub>405</sub> after 50 min.

### 3. Results

#### 3.1. Real-time RT-PCR assay development for *Tenacibaculum* spp.

The Tb\_rpoB assay was found to detect the common *Tenacibaculum* spp. strains isolated from diseased salmon in Northern Norway. It also detects *T. soleae*. The Tb\_tuf assay was found to be less specific and can detect a wider range of *Tenacibaculum* spp. including *T. ovolyticum* and *T. soleae*. Neither of these assays detects *M. viscosa*, *Aliivibrio* spp. and *Flavobacterium* spp. The efficacy of Tb\_tuf and Tb\_rpoB was 1.9397 and 1.9679 respectively based on RNA from a pure culture of *T. finnmarkense* strain HFJ<sup>T</sup>.

#### 3.2. Cohabitation experiment

Accumulated mortality during the cohabitation experiments is shown in Table 1 and Fig. 1. *T. finnmarkense* strain HFJ<sup>T</sup> caused 80% accumulated mortality in the shedders of both groups, but very low cohabitant mortality (Fig. 1). Diseased fish had classical signs of tenacibaculosis (Fig. 2): mouth erosion, frayed fins, and skin lesions. The smolts that died within the first few days had less clinical signs than the later ones; however all mortality showed some changes, in particular frayed fins. Histopathology of mouth and skin lesions showed large infiltrations of long thin rod-shaped bacteria within the dermis (Fig. 3). The bacteria recovered from the lesions were primarily pure cultures of *Tenacibaculum*-like bacteria, which were found to be genetically

identical to the challenge material (*atpD* gene).

Neither *T. finnmarkense* strain Tsp.2 nor *T. dicentrarchi* strain 35/09<sup>T</sup> caused an accumulated mortality in the shedders or the cohabitants that was greater than the control groups and very few fish showed clinical signs of tenacibaculosis. However, the bacteria that were recovered on MA and sequenced from these groups matched the challenge material. *Tenacibaculum* spp. isolates were recovered from the diseased control shedders and cohabitants, but these *Tenacibaculum* spp. were different through sequencing from the challenge material used in the experiment suggesting contamination from the water source.

The real-time RT-PCR screening of non-diseased cohabitants revealed that the skin and gills (except one heart tissue from a control fish) were the only tissues positive for *Tenacibaculum* spp. with the Tb\_rpoB assay (0–40% of screened fish). The Ct-values were high indicating low levels of *Tenacibaculum* (Supplementary Data). None of the sampled cohabitants showed clinical signs of tenacibaculosis, which may explain the lack or low presence of bacteria in the sampled tissues.

#### 3.3. Vaccine challenge

Accumulated mortality for each group in the vaccine challenge is presented in Table 2. There were no differences in mortalities between vaccinated and control fish in any of the groups, neither towards the homologous strain *T. finnmarkense* strain HFJ<sup>T</sup> nor towards the heterologous strain *T. finnmarkense* strain Tsp.2. Mortality in the control groups exposed to *T. finnmarkense* strain HFJ<sup>T</sup> was high, ranging from 90 to 100%, whereas the groups exposed to *T. finnmarkense* strain Tsp.2 ranged from 30 to 65%.

#### 3.4. ELISA

Results from the ELISA analysis are shown in Fig. 4. The data are presented as antibody response, visualizing the degree of binding of specific antibodies in the plasma towards the homologous *T. finnmarkense* strain HFJ<sup>T</sup>. Fish vaccinated with the high bacterial antigen content displayed a higher antibody response than fish vaccinated with the vaccine containing a lower content of bacterial antigen.

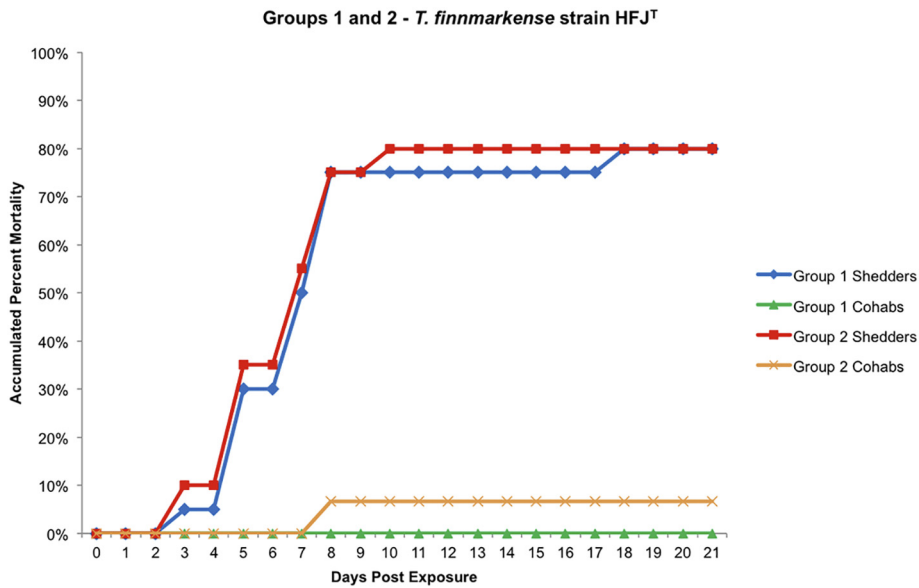


Fig. 1. Accumulated percent mortality for groups 1 and 2 from the cohabitation experiment. The shedders were bath infected with *T. finnmarkense* strain HFJ<sup>T</sup> at a bath concentration of  $4.9 \times 10^5$  cells mL<sup>-1</sup>.

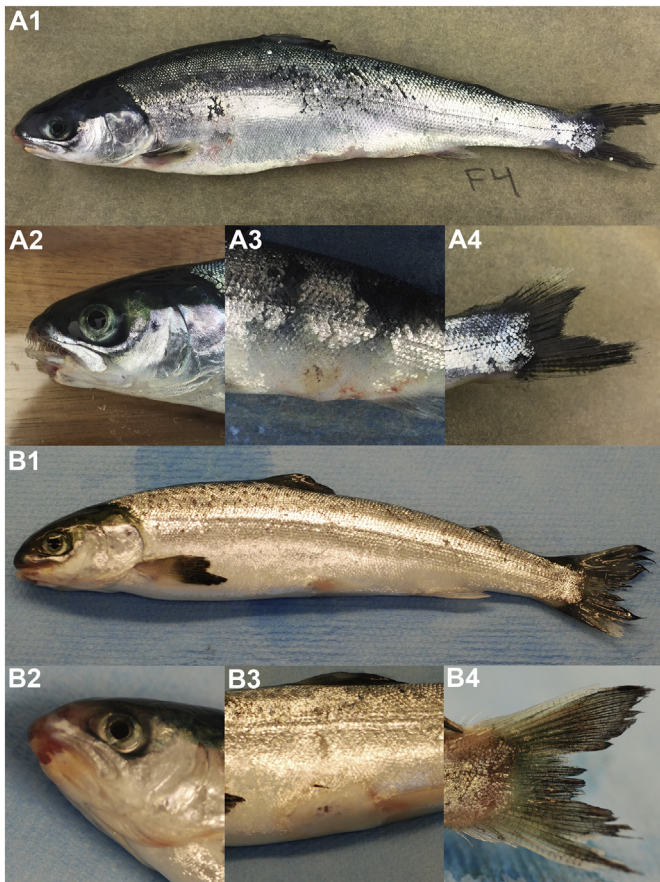


Fig. 2. A1: Diseased smolt from a natural tenacibaculosis outbreak at a Northern Norwegian salmon farm. B1: Diseased smolt from experimentally induced tenacibaculosis with *T. finnmarkense* strain HFJ<sup>T</sup>. Both fish show the classical signs of mouth erosion (A2, B2), skin lesions (A3, B3), and frayed fins (A4, B4) seen in tenacibaculosis.

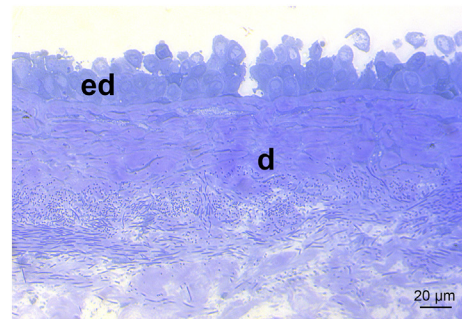


Fig. 3. Histopathology of a mouth lesion from a smolt experimentally infected with *T. finnmarkense* strain HFJ<sup>T</sup>. The epidermis (ed) is partially destroyed and an invasion of the dermis (d) with *Tenacibaculum*-like bacteria can be seen. There appears to be no associated host immune response. (Stained with Toluidine blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

#### 4. Discussion

This is the first report that shows that both *T. finnmarkense* strains used in this study induce tenacibaculosis through a bath infection of Atlantic salmon smolts without any pre-stressor or co-infection. This is in contradiction to what has previously been reported in bath challenge studies using Norwegian *Tenacibaculum* spp. (Olsen et al. 2011). One possible reason for this is the fact that there are differences in pathogenicity as shown by the differences in mortality rates between *T. finnmarkense* strain HFJ<sup>T</sup> and *T. finnmarkense* strain Tsp.2. Olsen et al. (2011) may have been testing strains that are not as pathogenic as *T. finnmarkense* strain HFJ<sup>T</sup>. Several studies have shown that *Tenacibaculum* spp. likely constitutes a part of the healthy microbiota of farmed Atlantic salmon (Karlsen et al. 2017b; Llewellyn et al. 2017); therefore a random *Tenacibaculum* spp. isolate recovered from a fish may not result in disease in challenge studies as the majority of these are likely not pathogenic. Due to the variability in pathogenicity between strains, a bath challenge model needs to be optimized for each strain. Other factors that would explain the differences in results between studies include the size of the fish, the smoltification status, and environmental conditions, which include water temperature and salinity. The difficulties in developing an effective and reproducible challenge model are

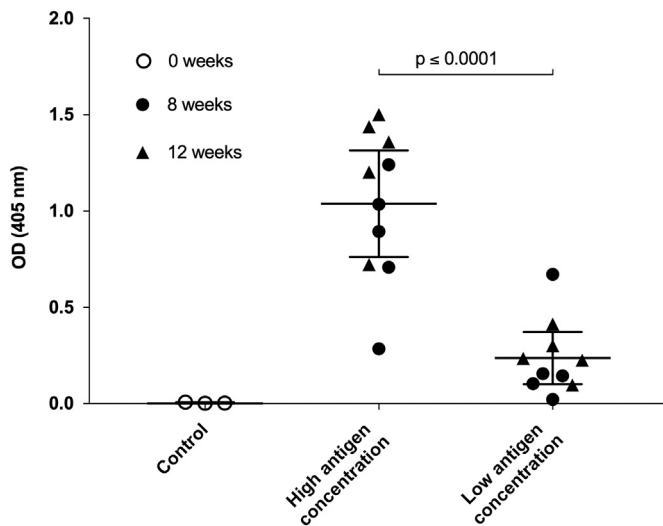


Fig. 4. Antibody response in plasma from fish vaccinated with high antigen concentration of *T. finnmarensis* strain HFJ<sup>T</sup> (1×) and low antigen concentration (0.06×) of *T. finnmarensis* strain HFJ<sup>T</sup> at 8 and 12 weeks post-vaccination, plotted as the OD (405 nm) value after 50 min incubation with substrate at the 1:100 dilution (blank, PBST, subtracted). The control is plasma sampled before vaccination (1:100 dilution). Statistical analyses were performed by use of GraphPad Prism 7. Antibody responses were analyzed using non-parametric Kruskal-Wallis one-way analysis of variance (ANOVA) test. Error bars indicate 95% confidence intervals (CI).

also seen with *F. psychrophilum*, a fish pathogen from the same family (Madetoja et al. 2000; Gómez et al. 2014; Long et al. 2014)

The general consensus is that smolts become less susceptible to pathogens the longer they have been exposed to saltwater. In a recent study by Småge et al. (2017), smolts that had been in the sea longer appeared to be less susceptible to tenacibaculosis. However, fish that were exposed to *T. finnmarensis* strain HFJ<sup>T</sup> in this study caused disease even after six weeks of saltwater adaptation.

This study is not the first report of a non-*T. maritimum* species causing tenacibaculosis in Atlantic salmon. In a study by Avendaño-Herrera et al. (2016) an isolate closely related to *T. dicentrarchi* strain 35/09<sup>T</sup> recovered from diseased Atlantic salmon in Chile was shown to induce ulcerative disease in both Atlantic salmon and rainbow trout, *Oncorhynchus mykiss*. In the current study, the *T. dicentrarchi* strain 35/09<sup>T</sup> only caused disease in a low level of smolts, indicating that *T. dicentrarchi* strain 35/09<sup>T</sup> is less pathogenic than *T. finnmarensis* strain HFJ<sup>T</sup> to Atlantic salmon smolts. In Norway, *T. dicentrarchi* is mainly recovered from diseased cleaner fish and asymptomatic Atlantic salmon (Olsen et al. 2017), which may indicate that this bacterial species may be more pathogenic to non-salmonid fishes.

The diseased smolts in these experiment presented similar clinical signs to what is seen during natural tenacibaculosis outbreaks in the field (Fig. 2), and seem to follow the same timeline with most of the mortality occurring one to three weeks post-transfer into saltwater (Kent 1992; Småge et al. 2017). The authors chose to conduct the experiments in 4 °C saltwater as this was the seawater temperature during the initial outbreak from which *T. finnmarensis* strain HFJ<sup>T</sup> was isolated. The low temperature may have contributed to the results, in particular with the vaccine challenge as metabolic rate would have been lower and therefore the immune system slower (Abram et al. 2017). Tenacibaculosis outbreaks in Northern Norway have occurred at higher temperatures (8 to 10 °C) with *T. finnmarensis* strain HFJ<sup>T</sup> being the most frequently recovered bacteria from the ulcers (Småge et al. 2017). In this study, the bacteria were grown in 16 °C for practical reasons, and the transition from 16 to 4 °C for the bath infection may have also contributed to the results by affecting the pathogenicity. More work is required to determine if temperature of both the culturing and

bath conditions has a significant impact on the course of the disease.

In the cohabitation experiment, there was low mortality in all groups including the controls, with the exception of the high mortality seen in the shedders exposed to *T. finnmarensis* strain HFJ<sup>T</sup> (Fig. 1), suggesting that there was some other stressor present. One possibility is the low temperature used. The MB that the control shedders were exposed to could have been the reason why those fish were susceptible to environmental bacteria; however, some control cohabitants were also affected and these were not exposed to MB. MB has been shown to be harmless to Atlantic salmon in other studies in quantities much higher than the ones used in this study (Olsen et al. 2011; Avendaño-Herrera et al. 2016).

The bacteria cause an external infection with very little to no systemic signs in most cases. The tissue screening of non-diseased cohabitants indicates that asymptomatic fish had a low prevalence of *Tenacibaculum* spp. externally and none internally. Tenacibaculosis is a very acute disease and bacteria may only be detectable internally towards the end when the tissue damage is sufficient for the bacteria to enter the blood stream. Further studies are required to investigate this. The lack of bacterial transfer from diseased fish to naïve ones seen in this study is also noted with *F. psychrophilum* (Madsen and Dalsgaard 1999).

Vaccination with *T. finnmarensis* strain HFJ<sup>T</sup> did not reduce mortality rates when challenged with the homologous strain. These results may not be surprising considering that *Tenacibaculum* spp. seem to induce little or no inflammatory response as seen in the histopathology (Fig. 3) and previously noted for the disease (Kent 1992). The rapidity of the infection may also contribute to the lack of protection, as the immune system may not have had enough time to respond. The immunization period of 1000 degree days was likely sufficient as the common protection period for commercial vaccines is 400 degree days and a vaccine against *M. viscosa* has been shown to induce protection after 600 degree days (Karlsen et al. 2017b). This study showed a good antibody response to the high antigen vaccine, further supporting a sufficient immunization period, which would indicate that this type of vaccine may not work for this bacterium. Although not included, analysis of mucosal antibodies may have been beneficial to determine whether or not a specific immune response had been induced.

Although the bath challenge model represents the natural route of infection, it is not the ideal way to test vaccine efficacy as the fish are exposed to one high dose of bacteria which may not reflect the natural infection pressure as suggested by Karlsen et al. (2017a). One way to avoid this would be to use a more controlled challenge model, such as intramuscular or IP; however these were tested in pre-studies with very poor results (data not shown). Another possible explanation is that the challenge dose for *T. finnmarensis* strain HFJ<sup>T</sup> may have been too high (reflected by the high mortality), which may have masked any potential effect of the vaccines.

Tenacibaculosis could become a larger problem than it is with the increased use of smolt facilities that pump in saltwater, which is unlikely to be completely sterile. This has the potential of introducing the bacteria into the facility as was seen in this study, where the controls of the cohabitation experiment were infected with what are likely strains of *Tenacibaculum* spp. (not *T. finnmarensis* strain HFJ<sup>T</sup> or Tsp.2, or *T. dicentrarchi* strain 35/09<sup>T</sup>) from the water source.

## 5. Conclusion

This study shows that *T. finnmarensis* strain HFJ<sup>T</sup> is a causative agent of tenacibaculosis as it presents in Northern Norway without the need for any scarification, pre-handling or co-infection. The pathogen does not appear to spread easily from fish to fish and more work is therefore required to determine possible reservoirs and/or vectors. Even though a whole cell inactivated vaccine induces an antibody response, it does not appear to offer protection against tenacibaculosis induced through a bath infection. Future developments need to include

finding the right protective antigen for a vaccine candidate and investigating the pathogen-host interactions.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.aquaculture.2018.06.063>.

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