Norwegian University of Life Sciences
Faculty of Veterinary Medicine
Department of Paraclinical Sciences

Philosophiae Doctor (PhD)
Thesis 2021:4

Bacterial impact on growth, health and disease of farmed Atlantic salmon and lumpfish

Bakteriell påvirkning på vekst, helse og sykdom hos oppdrettet laks og rognkjeks

Øystein Klakegg

Bacterial impact on growth, health and disease of farmed Atlantic salmon and lumpfish

Bakteriell påvirkning på vekst, helse og sykdom hos oppdrettet laks og rognkjeks

Philosophiae Doctor (PhD) Thesis

Øystein Klakegg

Norwegian University of Life Sciences
Faculty of Veterinary Medicine
Department of Paraclinical Sciences

Oslo (2021)

Thesis number 2021:4
ISSN 1894-6402
ISBN 978-82-575-1660-4

«Husk, du er støv! Husk, du er mer enn støv!»

Henrik Wergeland

Table of Contents

ACKNOWLEDGEMENTS

This study was carried out at Previwo AS in Averøy, Oslo and Solbergstrand and at the Department of Paraclinical Sciences at the Norwegian University of Life Sciences (NMBU) during the period of 2016-2020. The work was funded by Previwo AS and The Research Council of Norway (NFR) through their industrial PhD-program (Grant No. 260204/O30).

I would like to thank my excellent supervisor Henning Sørum at NMBU for ideas, discussions, strong support and sharing of his enormous knowledge, and for sharing the new discovery regarding bacteria in body tissues. Despite being continuously downloaded in work, he has always been available when needed. I am very glad that I, by coincidence, got in touch with him 17 years after we last worked together. It changed life.

Many thanks also to my co-supervisor Kira Salonius for guidance, support, profound knowledge, patience and generosity -and for tasty food, discussions, small walks and that you understood my English during stays in Oslo.

I wish to thank my wonderful colleagues at Previwo in Oslo and Solbergstrand and at NMBU at Lindern. Without you; Aud Kari, Camilla, Cristopher, Karoline, Gaute, Karla, Dhamo, Stanislav and Simen this work would not have been possible.

Thanks to all collaborating companies, Tjeldbergodden Rensefisk, Lumarine, Skorild, Njord and Pure Farming for access, knowledge, positivity, support and patience – and for lunches and necessary social contact.

Many thanks also go to co-authors for valuable contributions. Tommy thank you for data support when needed, and Sigurd, Valerie, Willa, and Johanne thank you for good help with my battle with the British language.

Barbo, Johanne, Sunniva, Maria and Åsmund thank you for your patience!

ABBREVIATIONS

16S *rRNA*	16S (Svedberg) ribosomal ribonucleic acid
AHL	(N)-acyl-homoserine lactone
AI	Auto-inducer
AMP	Adenosine monophosphate
BLAST	Basic Local Alignment Search Tool
cfu	Colony forming unit
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	Deoxyribonucleic acid
FCR	Feed conversion rate
GABA	γ-aminobutyric acid
GALT	Gut-associated lymphoid tissue
GIALT	Gill-associated lymphoid tissue
Lux	Bacterial luciferase
MALT	Mucosa-associated lymphoid tissue
MAMP	Microbe-associated molecular patterns
MLSA	Multi-locus Sequence Analysis
NALT	Nasopharynx-associated lymphoid tissues
NOK	Norwegian krone
OTU	Operational taxonomic unit
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
QQ	Quorum quenching
QS	Quorum sensing
RAS	Recirculating aquaculture systems
RT	Real-Time
SALT	Skin-associated lymphoid tissues
SGR	Specific Growth Rate
sp.	Species (singular)
spp.	Species (plural)
subsp.	Subspecies (singular)

SUMMARY

Despite considerable efforts to improve production with regard to animal welfare, disease control and sustainability in Norwegian aquaculture, both losses and feed conversion rate have not declined during the last 10 years. Lumpfish, used to control sea lice infestation in farmed Atlantic salmon, are lost to an even larger extent than Atlantic salmon. In both salmon and lumpfish, bacterial diseases are among the most important causes of death, and ulcer caused by bacteria contribute much to this.

At the same time, new knowledge in both mammals and fish regarding the interaction between many bacteria and hosts and the good, probiotic effects of some bacteria, have shown that bacteria are important for increasing robustness, disease resistance and growth. A better understanding of the interaction between bacteria and between bacteria and eukaryotes will probably help to make living conditions that promote health, growth and sustainability.

This thesis was initiated to investigate what impact adding potential probiotic bacteria to the two most abundant fish species in Norwegian aquaculture, lumpfish and Atlantic salmon has, and examine the effects regarding resistance to disease, survival and growth with an objective also to do some investigation regarding causality in bacterial diseases causing ulcer in Norwegian aquaculture.

Tenacibaculum dicentrarchi was isolated from a natural disease outbreak in post-smolts. The bacteria were characterized by bacterial cultivation and Multi-locus Sequence Analysis (MLSA). The disease was reproduced in a challenge trial. This is the first time that *T. dicentrarchi* has been shown to cause disease in salmon in Norway.

Bath treatment of Atlantic salmon by supplying probiotic *Aliivibrio* spp. (*A. njordis* NCIMB 42593 (VL1), *A. balderis* NCIMB 42592 (VL2) and *A. nannie* NCIMB 42594 (VL3)) to the rearing water indicated a significantly ($p < 0.05$) lower mortality, greater resistance to natural *Moritella viscosa* infection, less ulcers, faster growth and better feed conversion rate.

In lumpfish, bathing with the same probiotic bacteria indicated a significantly ($p < 0.05$) lower mortality, less ulcers, and increased resistance to *M. viscosa* infection.

The knowledge that bathing Atlantic salmon and lumpfish with probiotic *Aliivibrio* spp. results in lower mortality, increased resistance to some bacterial diseases and larger growth will probably advance animal welfare and sustainability in the aquaculture industry.

SAMMENDRAG (SUMMARY IN NORWEGIAN)

På tross av stor innsats for å bedre dyrevelferd og bærekraft i norsk lakseoppdrett, har ikke dødelighet eller fôrfaktor i norsk oppdrettsnæring blitt nevneverdig redusert de siste 10 år. Blant rognkjeks, som de siste årene er mye brukt for å redusere antall lakselus hos oppdrettslaks, er tapene enda større enn hos laks. Både hos laks og rognkjeks er sykdommer forårsaket av bakterier blant de viktigste årsakene til tap, og sår forårsaket av bakterier bidrar mye til dette. Ny kunnskap hos både pattedyr og fisk om samspillet mellom bakterier og vert og mange bakteriers gode, probiotiske egenskaper har samtidig vist at bakterier også er viktige for å bedre robusthet, sykdomsresistens og tilvekst. Når vi bedre forstår samspillet mellom bakterier og mellom bakterier og eukaryoter, vil vi være bedre i stand til å lage levemiljø som fremmer helse, vekst og bærekraft.

Dette PhD-prosjektet ble satt i gang for å undersøke hva slags effekt tilførselen av probiotiske bakterier til de to vanligste oppdrettsartene i Norge, rognkjeks og atlantisk laks, hadde, og for å se hvilken effekt det fikk på overlevelse, tilvekst og motstandskraft mot sykdom, og også undersøke årsaken til enkelte bakterielle sykdomsutbrudd med sår og forøket dødelighet hos oppdrettsfisk i Norge.

Tenacibaculum dicentrarchi ble isolert og karakterisert fra et sykdomsutbrudd på nyutsatt postsmolt ved hjelp av bakteriedyrkning og multi-lokus sekvensanalyse (MLSA). Sykdommen ble reprodusert i smitteforsøk. Dette er første gang *T. dicentrarchi* er påvist å gi sykdom hos laks i Norge.

Badebehandling med tilførsel av probiotiske *Aliivibrio* spp. (*A. njordis* NCIMB 42593 (VL1), *A. balderis* NCIMB 42592 (VL2) og *A. nannie* NCIMB 42594 (VL3)) i vannet til laks gav signifikant ($p<0.05$) lavere dødelighet, større motstandskraft mot naturlig *Moritella viscosa* infeksjon, færre sår, raskere tilvekst og lavere fôrfaktor.

Hos rognkjeks gav badebehandling med de samme probiotiske bakteriene signifikant ($p<0.05$) lavere dødelighet, færre sår og økt motstandskraft mot *M. viscosa* infeksjon.

Kunnskap om at bading av laks og rognkjeks med probiotiske *Aliivibrio* spp. gir lavere dødelighet, økt motstandskraft mot enkelte bakteriesykdommer og større tilvekst håper vi vil bidra til å bedre dyrevelferden og bærekraften i oppdrettsnæringen.

LIST OF PAPERS

Paper I

An outbreak of acute disease and mortality in Atlantic salmon (*Salmo salar*) post-smolts in Norway caused by *Tenacibaculum dicentrarchi*

Øystein Klakegg, Takele Abayneh, Aud Kari Fauske, Michael Fülberth, Henning Sørum
Journal of Fish Diseases, 2019 Jun;42(6):789-807. doi: 10.1111/jfd.12982.

Paper II

Enhanced growth and decreased mortality in Atlantic salmon (*Salmo salar*) after probiotic bath

Øystein Klakegg, Kira Salonius, Arve Nilsen, Michael Fülberth, Henning Sørum
Journal of Applied Microbiology, 2020 Jul;129(1):146-160. doi: 10.1111/jam.14649.

Paper III

Improved health and better survival of farmed lumpfish (*Cyclopterus lumpus*) after a probiotic bath with two probiotic strains of *Aliivibrio*

Øystein Klakegg, Siri Myhren, Rosemary Alice Juell, Marit Aase. Kira Salonius, Henning Sørum
Aquaculture, 2020, Volume 518, 734810.
https://doi.org/10.1016/j.aquaculture.2019.734810

1. INTRODUCTION

1.1 Fish farming, volume of production, losses and sustainability.

Worldwide

Since the first known cultivation of fish about 4000 years ago in China, aquaculture has grown tremendously and is today one of the most fast-growing food production systems worldwide (Stickney, 2009). In 2016 54.1 million tons of finfish were produced (FAO 1, 2018). This was an increase of 40.6 percent, 15.6 million tons, from 2010 (FAO 2, 2018). Freshwater finfish farming produced the largest quantum with 47.5 million tons or 87.8 percent of the total in 2016 (FAO 1, 2018). China is by far the largest aquaculture producer with more than 50 percent of the volume worldwide. Of the 369 different finfish species produced in aquaculture (including 5 hybrids) in 2016 carps were number 1, 2, 3 and 5 in volume; Grass carp (*Ctenopharyngodon idellus)* represented 6 068 000 tons, silver carp (*Hypophthalmichthys molitrix)* 5 301 000 tons, common carp (*Cyprinus carpio*) 4 557 000 tons and bighead carp (*Hypophthalmichthys nobilis*) 3 527 000 tons (FAO 2). Nile tilapia (*Oreochromis niloticus*), with 4 200 000 tons produced, had the fourth largest volume (FAO 2). Atlantic salmon (*Salmo salar*) represented the ninth largest species in quantity and contributed to the largest volume of fish produced in salt water, with 2 248 000 tons produced in 2016.

There has been a huge increase in finfish aquaculture production the last decades, with more than five times more produced in 2016 compared to 1990 (Figure 1).

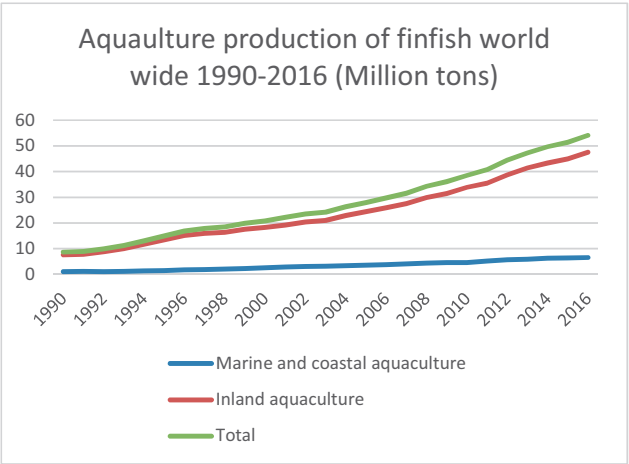

Figure 1. Aquaculture production of finfish worldwide per year from 1990 to 2016 in million tons. Figure made with numbers from FAO (FAO 2, 2018).

The annual growth has mostly varied between 4 to 16 percent (Figure 2).

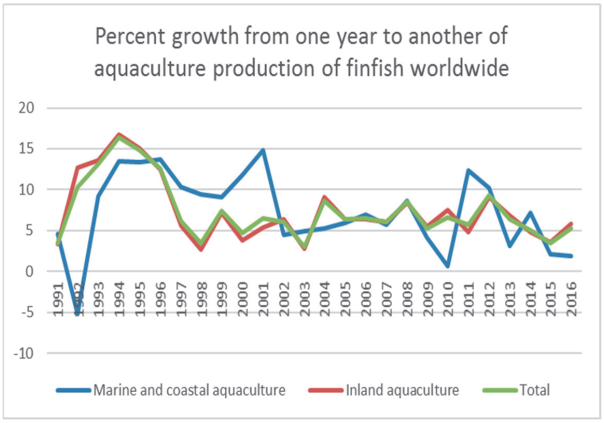

Figure 2. Growth of aquaculture production of finfish worldwide per year from 1991 to 2016 in percent. Figure made with numbers from FAO (FAO 2, 2018).

In 2016, the fish consumption per capita worldwide was 20.3 kg, an increase from 9.0 kg in 1960 (FAO 1, 2018). Due to an expected increase in the global human population to 9.8 billion in 2050 (United Nations, 2017), food production worldwide needs to increase 25-75% the next 30 years (Hunter, 2017).

Available land areal for agriculture is limited and capture fisheries has stagnated, so to meet the demand for food from the increasing and more protein eating global population, aquaculture production should increase, probably more than the average increase of food production (Ahmed et al., 2019; World Bank, 2013; FAO 2, 2018).

It is difficult to estimate losses and mortality in aquaculture worldwide, but despite of major improvements in the industry, not least related to vaccines, it is estimated that about 10 percent of all cultured aquatic animals are lost caused by infectious diseases worldwide with a value of more than 10 billion USD annually (Adams, 2019). In addition to losses due to infectious diseases, there are also huge losses due to other diseases, malnutrition, down-classification at slaughterhouses, escapes and quality of water. The total losses are estimated to be more than 20 percent.

As other feed/food production, aquaculture has environmental impacts. Use of land and water sites, pollution of water and sediments, diseases, use of feed and use of pesticides, chemicals and antibiotics represent some of the impacts on the

environment (United Nations, 2017). Reduction of the losses due to protection against bacterial diseases and improvements of the feed utilization rate, will result in a much better situation for both animal welfare and sustainability.

Norway

Norway has cultivated fish for a long time in freshwater. On a runestone in Oppland some 900 years ago the following script was carved: «Eilífr Elgr bar fiska í Rauðusjó» or Eilif Elg transported fish to the lake of Raudsjøen (Schøning, 1775). Norwegian commercial aquaculture with salmon and trout in salt- and brackish water started in the early 1950s (Osland, 1990; Tilseth et al., 1991). Since then the aquaculture industry has grown tremendously in Norway. The production value of aquaculture in Norway in 2017 was 65.0 billon NOK. Atlantic salmon, by far the most economically important species in Norway, contributed to 61.6 billion NOK (Statistics Norway, 2018). In 2017 Norway produced and sold 1 306 035 metric tons farmed fish, of which 1 236 353 metric tons was Atlantic salmon (Norwegian Directorate of Fisheries', 2019).

Figure 3. Today there are aquaculture sites along most of the coast in Norway. Photo Øystein Klakegg.

Although there is a great ambition in Norway to increase the production of salmon, actual production was reduced with 5.1 % in 2017 compared to 2015 (1 303 346 tons) (Norwegian Directorate of Fisheries', 2019). Despite considerable efforts to improve production with regard to animal welfare, disease control and

sustainability, both losses and production costs have increased. In 2017 there were 337.9 million Atlantic salmon smolt set out in cages in Norway. This was 23.1 million more than in 2016 (Norwegian Directorate of Fisheries', 2019). The number of losses of Atlantic salmon put to sea in Norway in 2017 was 57.8 million fish, compared to 56.0 million in 2016 and 43.6 million in 2014 (Norwegian Directorate of Fisheries', 2019). Dead fish (Atlantic salmon) constituted 49.5 million in 2017, 46.2 million in 2016 and 43.5 million in 2015. In 2017, 3.1 million Atlantic salmon were sorted out at the slaughterhouse.

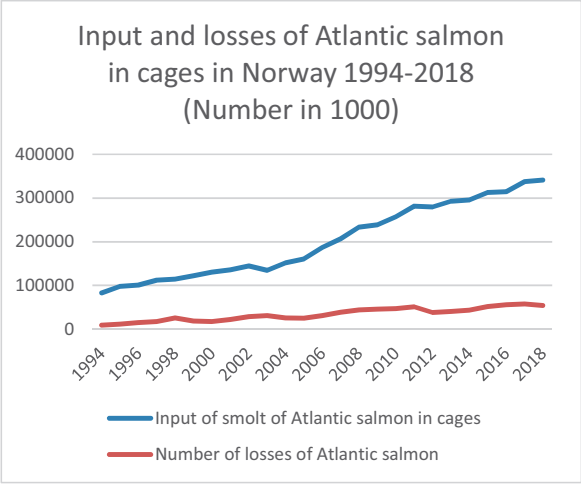

Figure 4. Number of Atlantic salmon put at sea and losses in Norway from 1994-2018. Numbers in 1000. Losses include dead fish, escapees, downgrading at slaughterhouses and other reasons. Figure made with numbers from Norwegian Directorate of Fisheries'.

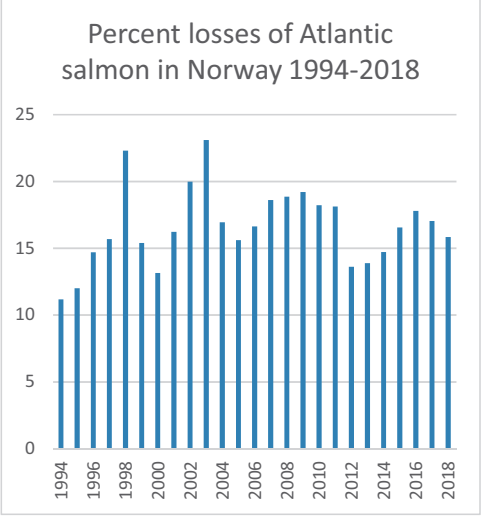

Figure 5. Percent losses of Atlantic salmon in Norway from 1994-2018. Losses include dead fish, escapees, downgrading at slaughterhouses and other reasons. The percent loss is calculated from the number of smolts put at sea the different years. Not all the fish that died were lost the year they were put at sea, so there is a minor bias in the figure.
Figure made with numbers from Norwegian Directorate of Fisheries'.

The cost of production per kg produced fish without the cost of slaughtering has increased significantly the last years. In 2017 the average cost of production was 30.74 NOK/kg, while in 2015 it was 26.15 NOK/kg, and in 2008 it was 18.61 NOK/kg (Norwegian Directorate of Fisheries', 2018). Although the cost of sea lice (*Lepeophtheirus salmonis*) treatments has increased, it is still the costs of feed that make up the largest single cost. The cost of feed was on average 14.38 NOK/kg produced salmon in 2017, compared to 9.93 NOK/kg in 2008. We must keep in mind that some of that cost also is feeding expense used to the 57.8 million salmon that were lost in 2017.

The Norwegian Directorate of Fisheries' profitability survey on the production of Atlantic salmon and rainbow trout revealed that the average economic feed conversion rate (eFCR) from 2008 to 2017 ranged between 1.21 and 1.35. The year 2017 was the second worst year on this metric, with an average eFCR at 1.32. The variation between sites and companies is considerable. In 2015 the best site had an economic feed conversion rate at 0.67 the worst had an economic feed conversion rate at 2.16 (Norwegian Directorate of Fisheries', 2018).

To control sea lice infestation in farmed Atlantic salmon the production of cleaner fish has increased tremendously the last years.

Figure 6. Production of farmed lumpfish has increased much the last years in Norway. Photo Øystein Klakegg

In 2008, 1.7 million farmed and wild cleaner fish were put into cages whereas in 2017 the number was 54.6 million (Norwegian, Directorate of Fisheries', 2019). Of the cleaner fish used in 2017 29.7 million were lumpfish (*Cyclopterus lumpus*), 11.2

million gold-sinny wrasse (*Ctenolabrus rupestris*), 7.7 million corkwing wrasse (*Symphodus melops*) and 2.4 million ballan wrasse (*Labrus bergylta*). Of these, 29.7 million lumpfish (all) and 0.95 million ballan wrasse were farmed, the rest were wild caught. Also, among the cleaner fish there are huge losses. The exact numbers of lost individuals are unknown, but losses as high as 100 % is suggested (Poppe, 2017). In a Norwegian survey from 2013, based on five sites and 79 000 lumpfish, an average of 48 % loss was reported, varying from 39 % to 100 % from site to site. Of the 13 864 dead lumpfish categorized regarding cause of death 75 % were categorized as bacterial infections (Nilsen et al, 2014). Sustainability is starkly questioned when half of the nearly 30 million lumpfish produced by farming are lost and with little use of the residual value, such as human consumption, after finishing their job as sea lice eaters.

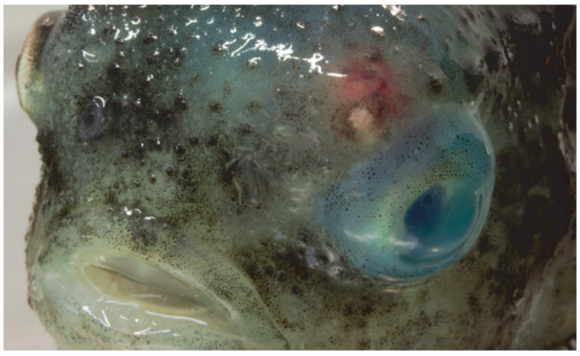

Figure 7. A diseased lumpfish. *Pasteurella* sp. was cultivated from lesions. Photo Øystein Klakegg

Until April 2018 it was required by law to vaccinate all farmed Atlantic salmon in Norway against furunculosis (*Aeromonas salmonicida* subsp. *salmonicida*), vibriosis (*Vibrio anguillarum*) and cold water vibriosis (*Aliivibrio salmonicida*). In addition, most salmon are also vaccinated against winter ulcers (*Moritella viscosa*) and many also against yersiniosis (*Yersinia ruckeri*). In some areas of Norway, the fish farmers are, from July 2020, required to vaccinate all Atlantic salmon and rainbow trout against pancreas disease virus (Nærings- og fiskeridepartementet, 2017). The mortality and losses of Norwegian farmed salmon have been significantly reduced since the first vaccines against vibriosis and against cold water vibriosis were introduced in the 1970s and in the 1980s, respectively (Gudding & Van Muiswinkel, 2013). Nevertheless, the losses from disease are still large in farmed fish compared to other livestock productions in Norway. Between 15 and 20 percent of the Atlantic salmon put to sea in Norway die, and infectious

diseases are the largest contributors to mortality prior to slaughter (Aunsmo, 2008; Mattilsynet, 2014).

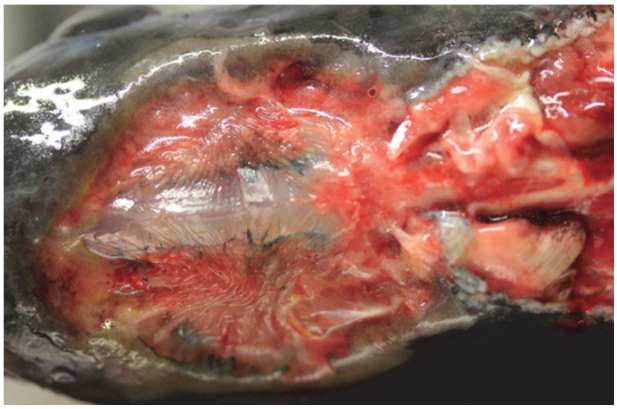

Figure 8. The last years between 50 and 60 million of the Atlantic salmon put at sea in Norway are lost (Norwegian, Directorate of Fisheries', 2019), most of them died, many of them because of infectious diseases. Many of these fish and fish sorted out at the slaughterhouse have suffered a lot. Photo Øystein Klakegg

Much of the loss today is due to chronic bacterial infections, often associated with ulcers from which the fish do not die in large outbreaks, but over a larger time scale (Hjeltnes et al, 2018). Although salmon are in the majority specifically vaccinated against winter ulcer (*M. viscosa*), a major part of the lost fish still die with large ulcers. Several bacteria that seem to play a role in the development of ulcers, disease and death among farmed fish are not given enough attention. This could partly be due to limited vaccine protection, and that bacteria other than *M. viscosa* also play a role in the development of ulcers.

Figure 9. Disease due to ulcers is one of the main reasons for losses and downgrading in Norwegian production of Atlantic salmon. From these ulcers we cultivated *Vibrio splendidus* and *Aliivibrio wodanis*. Photos Øystein Klakegg

It is not mandatory to vaccinate lumpfish in Norway, but most lumpfish are vaccinated against one or more of these agents: *Aeromonas salmonicida, Vibrio anguillarum, Vibrio ordalii, Vibrio splendidus, Vibrio logei, Aliivibrio wodanis, Moritella viscosa, Pseudomonas anguilliseptica* and *Tenacibaculum* (Vaxxinova, 2019).

1.2 **Microbiome and hologenome.**

The first visual observations of bacteria, or animalcules - "Latin animalculum= tiny animals" - were done by the Dutch scientist Antoni van Leeuwenhoek in 1667 (Haas, 1992). What Leeuwenhoek saw in his self-designed single-lensed microscope, and the importance of these microorganisms was not then known. As early as 1025 AD, the Persian polymath Ibn Sina, often known in the Western world as Avicenna, proposed a basic form of contagion theory (Has, 1991; Sarrafzadeh et al., 2001). Girolamo Fracastoro was in 1546 even more concrete in his *De contagion et contagiosis morbis*, long before any visualization of the causality, where he described a potential contagion as cause of syphilis (Gribanov & Potapchuk, 1996). Many other scientists after Leeuwenhoek paved the way for the acceptance of prevention of infection using hygiene practices (Baxby, 1999; Citrome, 2018; Rusnock, 2009; Walter, 1976). Among others are Edward Jenner, that in 1796 described how cowpox protects against smallpox, and Ignaz Philipp Semmelweiss that in 1847 showed how washing hands with chlorinated lime solutions prevented incidences of puerperal fever.

In the late 19th century Louis Pasteur and Robert Koch postulated the germ theory which states that microorganisms known as pathogens or germs can lead to disease. Since then and until recently bacteria were seen mostly as a source of illness, disease, decay and mortality (Mendelsohn, 2002). Today the importance of the bacteria is in process of changing from predominantly pathogens to mostly helpful agents (Ezenwa et al., 2012). It is generally accepted that eukaryote organisms from fungi to humans are inhabited by microbial organisms as bacteria and that most, if not all, eukaryote organisms are living in synergism with microbes (Bang et al., 2018; Esser et al., 2018; Morris, 2018). Some scientists are nevertheless claiming that some animal groups, among others a number of ants, caterpillars and shrimps, are harbouring few or none resident microbes (Hammer et al., 2019; Sanders et al., 2017).

The main conception today is nevertheless that the symbiosis of the eukaryote and the prokaryote is extremely important, and that intimate interactions between the host and the microbial community have made them functionally more or less as one unit, a metaorganism or a holobiont, the host and its microbiota (Guerrero et al., 2013; Rosenberg & Zilber-Rosenberg, 2011, 2018, 2019).

The hologenome concept, which considers the holobiont as an important level of evolution, was introduced in 2007 (Zilber-Rosenberg & Rosenberg, 2008). It suggests that some of the microbiome genome is transmitted together with the host genome from one generation to the next, and that genetic variations in the hologenome can be caused by changes in the host genome or in the microbiome genome (Rosenberg & Zilber-Rosenberg, 2011, 2018). The genetic changes in the host genome are slow, while the genome of the microbiome can change rapidly (Rosenberg & Zilber-Rosenberg, 2018). The change in the microbiome genome can occur by reducing or increasing microbial species, by acquiring new bacteria or by mutation or gene transfers (Morris, 2018; Rosenberg & Zilber-Rosenberg, 2018). It is suggested that the microbiome influences the fitness of its host and is an essential part of the host adaptability. (Bang et al., 2018). The research, so far particularly in gut, of microbiome and hologenome has revealed that the hosts often are inhabited with very complex microbial communities that have deep impact on the host (Bang et al., 2018; Dominguez-Bello et al., 2019).

Most of the work regarding microbiome and hologenome has been done with mammals, and the focus has been on the interactions between the host and its microbiota in the intestine (Esser et al., 2018; Marchesi et al., 2016) and in some degree on the skin (Ross et al., 2019).

The first research of microbiota in fish concerned intestinal and mucosal flora of haddock and was published in 1929 (Reed & Spence, 1929). As cultivation of salmonids became common in the 1970´s and 1980´s, studies of the salmonid microbiota also began (Yoshimizu et al., 1976; Yoshimizu, et al., 1976a, 1976b, 1976c, 1976d). In 1990 the first review of bacterial flora of fishes was published, when research of bacterial flora from eggs, skin, gills and intestines were summarized (Cahill, 1990). Different bacterial phyla as *Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria* and *Proteobacteria* are found in the gut of several different fish species (Llewellyn et al., 2014). Some of the findings were that

the microflora which was located in the gut seemed to originate from the environment or the feed and that the bacteria cultivated from the skin or gills could be transient rather than resident (Cahill, 1990). Water temperature, geographical location, salinity, developmental stages of the fish, diet and medical interventions such as antibiotics have all impact on the microbiota of the gut (Llewellyn et al., 2016; Wong et al., 2013; Zarkasi et al., 2014). The first review of the intestinal microflora of salmonids from 1995 similarly stated that the present bacterial genera vary with salinity, use of antibiotics, chromic oxide and diet (Ringø et al., 1995).

In Atlantic salmon as in other teleosts, the microbiome studied is mostly from the gut (Bakke-McKellep et al., 2007; Gajardo et al., 2016; Holben et al., 2002; Hovda et al., 2012; Ringø et al., 1995; Ringø et al., 2008; Zarkasi et al., 2014; Schmidt et al., 2016; Zarkasi et al., 2016; Zarkasi et al., 2017). A survey of wild Atlantic salmon from freshwater in eastern Canada and Ireland and seawater in the North Atlantic Ocean revealed that the microbiome in the gut of fresh water stages did not vary significantly between country of origin, but that the microbiome in the gut was significantly different between freshwater life-cycle stages and marine life-cycle stages (Llewellyn et al., 2016). In a survey of the gut microbiota of healthy post-smolt farmed Atlantic salmon in Norway, researchers found that the bacterial populations varied to some extent between the regions of the gut, proximal, mid and distal intestine, and much between digesta and the mucosa compartments. In digesta, *Proteobacteria* (Gram-negative) and *Firmicutes* (mostly Gram-positive), particularly lactic acid bacteria, were most abundant, while the mucosa-associated microbiota was dominated by *Proteobacteria* (Gajardo et al., 2016). A study in Norway looking for seasonal variation in the intestinal microbiota of Atlantic salmon performed by analyses of the genomic bacterial DNA from mid-gut and hind-gut did not identify seasonal variation (Hovda et al., 2012). In that study *Weissella cibaria, Lactobacillus fermentum* and *Lactococcus lactis* were the most common and consistent bacteria found throughout the year except in February when *Vibrio* spp. were predominantly found (Hovda et al., 2012). However, in a survey from Tasmania of the distal intestinal digesta of farmed Atlantic salmon with different diets they found that the species in the gut microbiome varied between the coldest and warmest months of the year. *Aliivibrio* spp. dominated all year, but they found more bacteroidia and clostridia bacteria during January and February, and more lactic acid bacteria from

February and onwards (Zarkasi et al., 2016). The sum of these results suggests that the microbiome of the gut of Atlantic salmon strongly reflects the environment.

It is important to reflect upon the method that has been used to determine the microbiome in the various studies. The first methods used cultivation of bacteria and biochemical reagents to determinate the bacterial species they cultivated. Then cultivation and 16S *rRNA* gene sequencing after secondary cultivation was often the selected method that was used. It has been known for a long time that perhaps less than 10 percent of the bacteria are culturable (Amann et al., 1995). As the methods to determine and find bacteria are being more and more sophisticated, less and less fractions of the known bacteria turns out to be cultivable. A work published in 2014 stated that cultivable microorganisms represent less than 0.1 percent of the total microbial community in the gut in some fish species (Zhou et al., 2014). This can result in a skewed bacterial study population, and to conclude and discuss findings based upon only a glimpse of the total picture of the diversity and amount of bacterial species in the microbiome should be done with caution (Zhou et al., 2014).

Today more and more of the microbial mapping is done by culture-independent metagenomic-sequencing, which provides a more total picture of the microbiota and much more information (Llewellyn et al., 2014; Llewellyn et al., 2016). The development of DNA extraction and high-throughput-sequencing techniques made the surveys and results of the salmon gut microbiome examination more accurate. The high-resolution map of the gut microbiota in Atlantic salmon done by Gajardo et al. confirmed older research and produced new knowledge (Gajardo et al., 2016). They found in total 914 operational taxonomic units (OTUs), clustered at 97 percent sequence identity (Gajardo et al., 2016). In the digesta in the gut Gajardo et al. found different phyla in relatively high concentrations: *Protebacteria* (47 %), *Firmicutes* (38 %), *Fusobacteria* (7 %) and *Actinobacteria* (6 %), whereas in the gut mucosa phyla *Protebacteria* (90 %) dominated, indicating that only a fraction of the microbes in the gut digesta can colonize the gut mucosa of the host (Gajardo et al., 2016).

It is also interesting that the phyla found in the gut intestine, both in Atlantic salmon and other species, are also the most common phyla colonizing the gut of terrestrial animals, such as humans (Gajardo et al., 2016; Looft et al., 2014; Sullam et al., 2012; Walter & Ley, 2011).

There are few surveys of microbial composition of mucus of healthy skin in Atlantic salmon, but in a survey from Norway it was found that mucus of healthy Atlantic salmon was mostly inhabited by residential *Tenacibaculum* spp. and *Arcobacter* spp. and that the bacteria species in the mucus were different from the bacteria in the surrounding water and sediments (Karlsen et al., 2017). The authors suggest that the mucus of Atlantic salmon contained a stable microbiota which could provide strong resistance against colonization. This is the same suggestion that was found in a survey of euryhaline fish, where the microbiome from homogenized carcass of the whole fish did not correlate with changes in the surrounding water. They found that deterministic processes probably drive fish microbiome assembly, not stochastic or random colonization (Schmidt et al., 2015). A survey of skin bacterial communities of European seabass (*Dicentrachtus labrax*) and gilthead seabream (*Sparsus aurata*) from Montpellier, France, demonstrated that the composition of the bacterial communities of the fish surfaces differ significantly from the bacteria in the surrounding water (Chiarello et al., 2015). They found that the microbiome of the skin was different between the two fish species and that skin bacterial communities varied among individuals and between body parts of the same individuals. Investigations of the microbiota of the skin in Atlantic salmon infected with the sea lice (*Lepeophtheirus salmonis)* revealed a reduction in microbial richness and a destabilization of the composition of the microbial community among infected fish (Llewellyn et al., 2017).

An investigation of skin microbiota of six fish species from the Gulf of Mexico revealed evidence for existence of specific skin microbiota associated with particular fish species (Larsen et al., 2013). The authors suggested that the composition of the skin microbiota could be considered as an active selection by the host. They found that *Proteobacteria* was the predominant phylum in skin biota from the six species they investigated, followed by *Firmicutes* and *Actinobacteria* (long Gram-positive bacteria).

The differences in the composition of the microbiome in gut and in skin, for instance; that the microbiome in the gut reflects the environment more both in quantitative terms and regarding bacteria species' composition than the microbiome in the skin – could be a result of a more active selection by the host. This

could indicate that microbiome of other areas of the fish is as important as the gut microbiome in some aspects of physiological functions, such as defence.

In lumpfish, we are not aware of any publications on the microbiome. Since the microbial colonization of the fish larvae, particularly the gut, is originating from the eggs, water and feed, the microbiota at least in the gut probably resembles other teleosts living in the same geographic water (Egerton et al., 2018).

1.3 **Interactions between bacteria.**

One of the first examples of intercellular communications within a bacterial population was found in *Streptococcus pneumoniae* by Tomasz (Tomasz, 1965). He found that competence, the physiological state in which bacteria are capable of taking up and undergo transformation by DNA molecules, was governed by an extracellular factor made by *S. pneumoniae* itself (Tomasz, 1966). Researchers later showed that *Aliivibrio (Vibrio) fischeri* and *Vibrio harveyi* produced light in high cell density populations but not in dilute suspensions (Nealson et al., 1970). Ten years later the luminescence genes *luxI* and *luxR* that control lux gene transcription from *A. fischeri* were identified together with the auto-inducer, the Quorum Sensing signal (Eberhard et al., 1981; Engebrecht et al., 1983; Engebrecht & Silverman, 1984).

Quorum sensing (QS) is now accepted to be one of the most important intercellular communication between bacteria. It works mainly like this: as a population of bacteria grows, the bacteria secrete small signal molecules, called auto-inducers. The extracellular concentration of the signal molecules increases, and when the threshold concentration is reached, the bacterial cell group detects the signal molecule and responds as a population. Quorum sensing is the ability for a bacterium to detect and respond to the density of bacterial cells by regulation of genes.

The Quorum sensing in *A. fischeri* is an example of how bacterial genes and auto-inducers function. The *LuxI* gene, in the *luxICDABE* operon of *A. fischeri* initiates the production of an auto-inducer, a Quorum Sensing signal (QS-signal), which is excreted from the bacterium. This auto-inducer interacts with the product of the *LuxR* gene in other nearby bacteria when it reaches a large enough intra-bacterial concentration and make a *LuxR*-auto-inducer complex. This *LuxR*-auto-inducer complex is a transcriptional activator of the *luxICDABE* operon. The *luxICDABE*

operon activation establishes a positive feedback loop which increases the level of auto-inducer production, via *luxI*, and the amount of light via *luxCDABE*.
While upregulating the *luxICDABE* transcription, the *luxR*-auto-inducer complex acts by downregulating the *luxR* transcription. These two autoregulatory loops therefore control light production by *A. fischeri* in response to the auto-inducer concentration which is a product of the concentration of bacteria (Eberhard et al., 1981; Engebrecht et al., 1983; Engebrecht & Silverman, 1984).

The QS-signal produced via the *luxI* gene expression in *A. fischeri* is an N-3-oxohexanoyl-L-homoserine lactone (3OC6-HSL), an N-acyl-homoserine lactone (AHL) (Eberhard et al., 1981). The AHL QS-signal is sometimes referred to as auto-inducer-1 (AI-1). This is a common feature of Gram-negative QS-bacteria, which often communicate with an AHL QS-signal (Bassler & Losick, 2006). This is in contrast to some Gram-positive bacteria, such as *Bacillus* and *Streptococcus*, which often communicate with oligopeptides (Bassler & Losick, 2006; Okada et al., 2005). The QS-signal, the small peptide, of many Gram-positive bacteria is recognized by the bacterium with the help of a membrane-bound histidine kinase. Inside the bacteria the oligopeptide initiates, by phosphorylation, a cascade that impinges DNA binding transcription factors responsible for target genes regulation (Bassler & Losick, 2006). Both the oligopeptide-QS and the AHL-QS are often unique for each bacteria species which produce their own special AHL or oligopeptide (Bassler & Losick, 2006).

Several other QS-systems have been discovered in the latest years, particularly in Gram-negative bacteria, for instance quinoline based QS-systems and thiazole-carbaldehyde based QS-systems (Lee & Zhang, 2015; Lin et al., 2018; Wang et al., 2019; Zhang et al., 2018). Some QS-signals are found in both Gram-negative and Gram-positive bacteria, as for instance the furanosyl borate diester, called auto-inducer-2 (AI-2) (Beeston & Surette, 2002; Chen et al., 2002; Schauder et al, 2001).

The same bacteria can have many different QS-systems. Thus, the former description of *A. fischeri luxI/luxR* system is a simplification. The expression of *A. fischeri* luminescence genes also needs a cyclic AMP (cAMP) receptor protein (CRP) with cAMP to act properly. Both the auto-inducer-LuxR complex and the cAMP-CRP complex function as transcriptional antagonists in the luminescence gene regulation in *A. fischeri* (Dunlap & Greenberg, 1985, 1988). In addition, there are several

different N-acyl-homoserine lactone (AHL) QS signal pathways that affect the *luxICDABE* in *A. fischeri* (Hao et al., 2010).

The metagenomic DNA libraries that now are made reveal that many bacteria have several distinct QS-systems (Girard, 2019; Hao et al., 2010). One example is *Pseudomonas aeruginosa* which has at least four different QS systems (Lin et al., 2018). One of them, the quinoline signal, which stimulates some virulence genes is extremely hydrophobic, and must be packed and transported into vesicles derived from the bacterium's own membrane through the aquatic environment surrounding the bacteria cell before it reaches the neighbour cells where the bacterial derived membrane vesicle delivers its QS-molecules (Lee & Zhang, 2015; Mashburn & Whiteley, 2005; Pesci et al., 1999). There is also an indication that this QS-molecule additionally functions to mediate membrane vesicle biogenesis and iron acquisition and communicates to its eukaryote host (Lin et al., 2018).

The same membrane derived vesicles from *P. aeruginosa* is in addition an example of intercellular communication between different species of bacteria. These vesicles can also contain other quinolines that act as antibiotics and can kill bacteria of other species when delivered to them (Bassler & Losick, 2006).

An example of intercellular communication between different bacteria species where the recipient has utilized the QS-signal is *Salmonella typhimurium* which possesses a *luxR* homolog, but does not have a *luxI* type gene and does not produce AHL (Ahmer et al., 1998; Smith & Ahmer, 2003). Instead *S. typhimurium* responds to AHLs produced by other species of bacteria which activate specific genes in *S. typhimurium* (Smith & Ahmer, 2003).

To simplify: the quorum sensing signals are N-acyl-homoserine lactones (AHLs) that are found in *Proteobacteria* (Gram negative bacteria) , the oligopeptides are found in *Firmicutes* (Gram positive bacteria) and auto-inducer -2 (AI-2) are found in both in *Proteobacteria* and *Firmicutes* (Bassler & Losick, 2006; Mayville et al., 1999; Xavier & Bassler, 2005a, 2005b).

Another question is how far away from each other two aggregates of bacteria can be while still communicating, and 10-100 μm is regarded as the longest distance of communication between aggregates (Boyer & Wisniewski-Dye, 2009; Connell et al., 2014; Decho et al., 2011).

These cell-to-cell signalling phenomena found by Tomasz, Nealson, Eberhard, Engebrecht and others were for many years considered to be rare and restricted to a few species. Today we know that cell-to-cell communication is very common and occurs in both Gram positive and Gram negative bacteria and most likely in the majority of bacteria (Whiteley et al., 2017).

Co-infection is not a seldomly occurring circumstance, nor is it in fish, and communication occurs between the different bacterial species (Kotob et al., 2016). Two of the examples that act synergistically during co-infection are *Renibacterium salmoninarum* and *Aeromonas hydrophila* in chinook salmon (*Onchorhynchus tshawytscha*) and *Edwardsiella ictaluri* and *Flavobacterium columnare* in Thailand striped catfish (*Pangasianodon hypophthalmus)* (Crumlish et al., 2010; Dong et al., 2015; Loch et al., 2012). *Aliivibrio wodanis* and *Moritella viscosa* act antagonistically during co-infection in Atlantic salmon (Hjerde et al., 2015; Karlsen et al., 2014). The inhibiting effect *A. wodanis* has on *M. viscosa* could either be of AHL-QS or most probably due to a bacteriocin agent (Hjerde et al., 2015). Another example is the afore mentioned luminescent *V. harveyi* that can react to a self-produced signal or a signal produced by other bacterial species to produce light (Nealson et al., 1970). An additional example of how different bacteria may benefit from each other is in biofilms with bacterial clusters attached to surfaces encased in extracellular polymeric matrix. Biofilms have often enhanced tolerance to antimicrobial compounds (Crabbe et al., 2019).

It is now accepted that intercellular communication is the norm in the bacterial world and that QS is involved in regulation of various features like: virulence, bioluminescence, antibiotic production, motility, swarming, biofilm formation, sporulation, symbiosis and growth inhibition (Bassler & Losick, 2006; Mukherjee & Bassler, 2019).

Anti-quorum strategies for counteracting quorum sensing bacteria, so called quorum quenching (QQ) mechanisms have evolved. This is achieved by inactivating the QS-signal, by degrading the QS-molecules, by blocking their receptors by mimicking the QS-molecules or by modification of the QS-signals by enzyme activity. Examples of QQ are for example reported in different bacteria in Red Sea sediments that can degrade AHL-QS molecules produced by *P. aeruginosa* and inhibit biofilm formation from *P. aeruginosa* and quorum quenching bacteria used to degrade AHL-

QS in osmosis membranes and thereby inhibit biofouling (Oh et al., 2017; Rehman & Leiknes, 2018). Another example of QQ is the degradation activity *Tenacibaculum* sp. strain 20J has on *Edwardsiella tarda* AHL production (Romero et al., 2014).

In addition to QS-signals affecting other bacteria, many bacteria also produce and excrete bacteriocins and antibiotics affecting other bacterial species as mentioned for *A. wodanis* and *P. aeruginosa* (Bassler & Losick, 2006; Hjerde et al., 2015). *Streptomyces* represent another example of socially mediated induction and suppression of other bacteria during bacterial co-existence excreting for instance antibiotics (Abrudan et al., 2015).

The social interaction between bacteria and the resulting synchronized behaviour enables the bacteria to act like a multi-cellular organism (Bassler & Losick, 2006; Camilli & Bassler, 2006; Fujimoto et al., 2018; Rader & Nyholm, 2012). Bacteria are socially communicating organisms that modulate many behaviours important for reproductive success (Whiteley et al., 2017). The words Edward Osborne Wilson, the father of sociobiology, wrote regarding humans, could also be written regarding bacteria: «Competing is intense among humans, and within a group, selfish individuals always win. But in contests between groups, groups of altruists always beat groups of selfish individuals."

1.4 Interactions between bacteria and eukaryotes.

For decades bacteria were mostly seen as negative and only harmful in general. Humans have known for centuries that something small could make trouble, long before the knowledge about bacteria existed. For instance, Norwegians shot *Clostridium* contaminated arrows by crossbow or spears into whales at the coast of Norway hundreds of years ago, causing gangrene and septicaemia to the whales and made them easier to kill or led to beaching (Øen, 1996). Norwegians had introduced laws determining the ownership of a whale carcass as early as the 10th century AD (Read, 2013).

Most pathogenic bacteria feature virulence factors which either are toxic (toxins) causing an interaction directly to host tissues or by producing signalling proteins that elicit a host response helping the pathogenic bacterium in colonization or replication (Diard & Hardt, 2017; Uzman, 2003). There are many different types of toxins, and these are categorized in two major groups: the endotoxins and exotoxins. The endotoxins are part of the lipopolysaccharide of the cell wall of Gram-

negative bacteria, and exotoxins are secreted proteins of both Gram-negative and Gram-positive bacteria (Mongeau, 1981; Popoff, 2018). In addition the bacteria have other virulence factors, some directly injected into the eukaryote cell when the bacterium is attached to it (Popoff, 2018).

In the rest of this chapter the focus will be on the microbiota and probiotic bacteria and how the bacteria interact with the host. Bacteria have been used for fermentation and potential positive effects for nearly 10 000 years, for instance in yoghurt and kefir (Aryana & Olson, 2017; Ozen & Dinleyici, 2015). The Russian Nobel prize winner in medicine, Ilja Metsjnikov, is often referred to as the father of probiotics. In his book published in 1907: "*The Prolongation of Life"* he suggested that by intake of microorganisms one could modify the gut flora and that this could have significant health benefits. The word probiotic comes from Greek "pro bios" and means "for life". The word probiotic was first used in 1965 (Lilly & Stillwell, 1965). In the beginning the term was used to describe a microorganism or a product of a microorganism that stimulated the growth of another. Food and Agriculture Organization of the United Nations (FAO) and the World Health Organisation (WHO) definition: "live microorganisms which when administered in adequate amounts confer a health benefit on the host", is today the most accepted definition of a probiotic (Hill et al., 2014). To guidance stakeholders the International Scientific Association for Probiotics and Prebiotics (ISAPP) made in 2014 a consensus statement on the scope and appropriate use of the term probiotic guidelines for defining and using probiotics (Hill et al., 2014). Evidence of health benefit is required to be called a probiotic. Probiotic may have different routes of administrations, both humans and animals may be the target and probiotics must be safe for their intended use. Undefined consortia such as faecal microbiota transplant, fermented food with undefined microbial content, dead microbes and microbial products and components do not come under the probiotic definition (Hill et al., 2014). The definition of probiotics also includes adequate amount of the administered probiotic. The quantity of probiotic bacteria has to be sufficient to confer health benefits to the host. It is difficult to make a universal rule of amount of administration to be defined as a probiotic, but both Italia ($>1X10^9$ cfu/ per serving) and Canada ($1X10^9$ cfu/day) have quantified the number of bacteria delivered in food (Health Canada, 2009; Ministero della Salute, 2013)

The body of a fish, a mollusc or a human is a fine and wonderful instrument in which the different organs communicate and interact in a sophisticated and complex manner. The eukaryote-prokaryote interaction is probably one of the answers to how sophisticated and fine-tuned the eukaryote appears (Ezenwa et al., 2012). It is known that some of the quorum-sensing signals from the bacteria, auto-inducers, can act like hormones and affect cells in their eukaryotic host, and opposite that hormones from the eukaryotic host are able to modulate the prokaryotic bacteria (Bassler & Losick, 2006; Pacheco & Sperandio, 2009). The co-evolutions of the bacteria and the host, and the communication and interaction between the two, are yet to be fully understood. It is now accepted that eukaryotic organisms live in interdependent relationships with their microbiome (Esser et al., 2018).

One of the first examples of the close relationship discovered between a bacterium and an eukaryote is that of *Aliivibrio fischeri* (former *Vibrio fischeri*) and the Hawaiian bobtail squid (*Euprymna scolopes*), where the squid in exchange for a home and a supply of carbohydrates and amino acids to the bacterium benefits from illumination produced by the bacteria to hide the squid`s silhouette and avoid predators (Ezenwa et al., 2012; Rader & Nyholm, 2012). The squid has small sacs, the light organ, on its underside. When the squid is born the light organ is sterile. The light organ is selectively colonized by *A. fischeri*, which only occur in low numbers in the seawater surrounding the squid (McFall-Ngai, 2014; Whiteley et al., 2017). Inside this light organ *A. fischeri* acquires protection and a nutrient. The bobtail squid is active mainly at nights. During daytime it is mainly sitting still in the sand, camouflaged by its exterior. During night-time it is swimming and hunting. Then the squid could cast a shadow from the moon or the starlight to reveal it to predators underneath. They do not because the *A. fischeri* produce light from inside the light organs, resembling moon and starlight, and as a result, the bobtail squid is not seen by their predators. In the morning, when daylight occurs the squid vents about 90 percent of the bacteria back into the seawater. At this stage when sitting still on the sand the rest of the *A. fischeri* in the light organ eat and reproduce, so the squid the next evening and night will have a full complement of luminous *A. fischeri*. As mentioned earlier, the society of the bacteria produce light as a group only when the amount of autoinducer is over the threshold value (McFall-Ngai, 2014; Rader & Nyholm, 2012). The symbiosis of *A. fischeri* and the squid is one of the most studied,

and the knowledge expands further. Now we also know that the presence of *A. fischeri* in the light organ also influences the development of the eye function, such as lens formation, phototransduction and visual perceptions of the squid (Backhed, 2019; Moriano-Gutierrez et al., 2019). Our understanding of the interaction between host and microbiota is constantly evolving.

Another example of the interaction between the host and the microbiota is that mice (*Mus musculus*) fed with probiotic *Lactobacillus rhamnosus* have higher expression of the γ-aminobutyric acid (GABA) receptor in the brain and exhibit decreased anxiety and better performance in stressful situations (Bravo et al., 2011). Bravo et al. found evidence that the vagus nerve was the major communication pathway between the bacteria in the gut and the brain (Bravo et al., 2011). Bacteria could also modify neural and endocrine activity in the brain of their host, explaining some of the large implication of the power of bacteria and also how bacteria can influence the social behaviour of its host (Bravo et al., 2011; Ezenwa et al., 2012). These examples also indicate that animals actively can control their symbiont population (squid) and that bacteria also can alter the mood of their host (mice). The current knowledge about the fish host-microbe interactions is emerging.

The bacteria and their host communicate mainly with each other through different hormonal signals (Bassler & Losick, 2006; Hooper et al., 2012; Hughes & Sperandio, 2008; Pacheco & Sperandio, 2009; Semova et al., 2012). The QS molecules play an essential role in the host-microbial symbiosis (Hughes & Sperandio, 2008; Li, Ren, & Fu, 2019). It is evidence that AHL (AI-1), the oligopeptides, as well as the AI-2 and also the aromatic auto-inducers (AI-3) can perform communication between bacteria and the eukaryote (Hughes & Sperandio, 2008; Li et al., 2019; Pacheco & Sperandio, 2009). In addition, some the interaction between bacteria and the host are likely mediated via host immunity improving resistance to bacterial infections (Taoka et al., 2006).

The effects of the bacterial auto-inducers (AIs) on the vertebrate host's immune system have been studied. For instance, there have been reports that the activity of neutrophils, macrophages, and T-lymphocytes can be modulated by the bacterial AIs (Belkaid & Hand, 2014; Hughes & Sperandio, 2008; Li et al., 2019; Pacheco & Sperandio, 2009).

It is also largely the communication between the gut microbiota and the host that has been studied so far (Baumler & Sperandio, 2016; Belkaid & Hand, 2014; Butt & Volkoff, 2019; Dimitroglou et al., 2011; Dominguez-Bello et al., 2019; Kostic et al., 2013; Li et al., 2019; Marchesi et al., 2016; Rawls et al., 2006; Roeselers et al., 2011; Sekirov et al., 2010; Suez et al., 2018; Walter & Ley, 2011). Some of the effects the gut microbiota has on its host, in addition to provide nutrients, are protecting against pathogens and shaping of the immune system (Backhed, 2019; Brugman et al., 2018; Rawls et al., 2006).

The interaction between bacteria and fish is not so well documented as the interaction between bacteria and mammals. It is particularly the Zebrafish *(Danio rerio)* that has been the model for how commensal microorganisms inhabit every mucosal surface of teleost fish (Kelly & Salinas, 2017). Colonization of the mucosal surfaces by beneficial bacteria can prevent colonization of pathogenic bacteria through competitive exclusion or production of antimicrobial compounds (Boutin et al., 2012; Brugman et al., 2018; Carbajal-Gonzalez et al., 2011). The microbiota has impact on the development and functions of the teleost's immune system (Kelly & Salinas, 2017). In particular the mucosa-associated lymphoid tissues (MALTs) have been investigated (Kelly & Salinas, 2017). Teleost fish have four different MALTs, the gill-associated lymphoid tissue (GIALT), the gut-associated lymphoid tissue (GALT), the nasopharynx-associated lymphoid tissue (NALT) and the skin-associated lymphoid tissue (SALT). These mucosal surfaces are covered by a continuously produced mucus layer that contains immunologically important molecules that interact with the commensal microbial populations at the mucosal surfaces. Mucosal infections in teleosts can alter both the amount and the contents of the mucus (Estensoro et al., 2013; van der Marel et al., 2010).

Vibrio spp. and *Aliivibrio* spp. are some of the most common bacteria in seawater and are often associated with fish. As seen in the symbiosis with the bobtail squid, they can react intimately with their host, or with other bacteria and very many are producing QS-signals to interact with other bacteria or eukaryotes. Of 61 species of *Vibrio/Aliivibrio* tested for AHL production, 73 percent produced it and out of 48 species tested for AI-2 production, 85 percent were positive (Girard, 2019). The common occurrence of these QS-signal systems indicates that many of the

Vibrio/Aliivibrio bacteria interact frequently with each other and possibly with the host.

1.5 Probiotic bacteria in aquaculture

The term probiotic in aquaculture has historically been used more broadly than the FAO/WHO definition and often included both live bacteria, bacterial products and dead bacteria and their effects of stimulation of growth and improved water quality (Newaj-fyzul et al., 2013, Reddy, 2014, Tuan et al., 2013). In aquaculture the first known use of probiotics was in 1986 by M. Kozasa who added *Bacillus toyoi* as growth promotor of the Japanese amberjack (*Seriola quinqueradiata*) (Kozasa, 1986).

Since then, probiotic bacteria have been applied to many fish species, among others: cod (*Gadus morhua*), turbot (*Scophtalmus maximus*), Senegalese sole (*Solea senegalensis*), rainbow trout (*Oncorhynchus mykiss*), gilthead seabream (*Sparus aurata*), European bass (*Dicentrarchus labrax*), European eel (*Anguilla anguilla*) and Atlantic salmon (Akhter et al., 2015; Balcazar et al., 2006; Feckaninová et al. 2017; Hoseinifar et al., 2018; Kuebutornye et al. 2020; Martinez Cruz et al., 2012; Tapia-Paniagua et al., 2014; Verschuere et al., 2000; Zaineldin et al., 2018). We are not aware of any scientific report of probiotic bacteria applied to lumpfish.

More than one hundred distinct bacterial species are used as probiotics for fish. Among those are the following: *Aeromonas* spp., *Bacillus* spp., *Carnobacterium* spp., *Lactobacillus* spp., *Micrococcus* sp., *Pseudomonas* spp., *Shewanella* sp., *Streptococcus* spp. and *Vibrio* spp. (Akhter et al., 2015; Martinez Cruz et al., 2012; Merrifield et al., 2010). Probiotic administration to fish has often been given to fish larvae and have among other prevent mortality and increased growth (Lauzon et al., 2010; Villamil et al., 2010). Several *Vibrio* spp. have been considered for use as probiotics in aquaculture. *Vibrio alginolyticus* inhibits *V. ordalii*, *V. anguillarum*, *A. salmonicida* and *Yersinia ruckeri*, and protected Atlantic salmon to some extent against *V. ordalii*, *V. anguillarum* and *A. salmonicida.* (Austin et al., 1995). V. *fluvalis* protected rainbow trout against *A. salmonicida* (Irianto and Austin, 2002) and *V. proteolyticus* was found to improve protein digestion in juvenile turbot (DeSchrijver and Ollevier, 2000).

Many bacteria, both Gram-positive and Gram-negative, have been tested as candidates for probiotics also to Atlantic salmon, amongst them are: *Carnobacterium inhibens, Carnobacterium divergens, Lactobacillus delbrueckii, Tetraselmis suecica, Pseudomonas fluorescens* and as mentioned *V. alginolyticus* (Austin et al., 2006; Gram et al., 1999; Irianto & Austin, 2003; Ringø et al., 2007; Salinas et al., 2008).

The documented effects of the probiotic bacteria vary from a source of nutrients and enzymatic contribution to digestion, improved growth of the host, improved water quality, enhanced immune response or antiviral effect, improved stress tolerance, improved reproduction in the host, inhibition or competitive exclusion or weakening of pathogenic microorganisms, reduced malformations, improved gut morphology and improved microbial balance in the gut (Banerjee & Ray, 2017; Martinez Cruz et al., 2012; Merrifield et al., 2010; Mohapatra et al., 2013).

As mentioned in sections 1.3 and 1.4 the mode of actions of the probiotic bacteria against other bacteria often is via QS-systems that regulate among others virulence, growth inhibition, antibiotic production and motility or via bacteriocins and antibiotics which directly affect other bacterial species (Abrudan et al., 2015; Bassler & Losick, 2006; Hjerde et al., 2015; Mukherjee & Bassler, 2019). The antagonistic activity against other bacterial species could also be competition for nutrient uptake, competition for iron uptake, competition for colonization sites and immune modulation via the host (Banerjee & Ray, 2017; El-Kholy et al., 2014). Many probiotic bacteria also produce extracellular enzymes as lipase, amylase and protease to support the host metabolism process (Krogdahl et al., 2005; Liu et al., 2016). Some probiotic strains also perform immune modulation of the host often by influence the innate or adaptive immunity by production of cytokines such as interleukins (Foey & Picchietti, 2014; Nayak 2010)

Historically, probiotics for fish have mainly dealt with feed and the gut (Akhter et al., 2015; Dimitroglou et al., 2011; Falcinelli et al., 2015; Lazado & Caipang, 2014; Nayak, 2010; Suez et al., 2018).

Although the far most common way of application of probiotic bacteria to fish has been to apply it together with the feed, some probiotic treatment have been done by adding probiotic bacteria to the rearing water, mostly for improving the water

quality but also for improved growth and better survival (Hai, 2015a, 2015b; Molina et al., 2009).

1.6 Bacteria causing ulcers in Atlantic salmon and lumpfish

Ulcers are a large problem for both Atlantic salmon and lumpfish. In Norway it is estimated that more than 2.5 % of the Atlantic salmon put at sea die or are downgraded at the harvest plant due to ulcers (Takle et al., 2015). In a survey in 2008 of 2088 dead Atlantic salmon in Norway the investigators found that ulcers were the main cause of death, accounting for 43 % of the assigned mortality, and also that infectious agents were involved in 64 % of the total mortality (Aunsmo et.al., 2008). The numbers of lumpfish diseased or dead caused by ulcers are not known, but in a Norwegian survey from 2014, 75 % of the mortality in lumpfish were classified as caused by bacterial infections, in which some may cause ulcers, this in addition to 1 % classified dead by ulcers or rotten fins (Nilsen et al., 2014).

Atlantic salmon

Vibrio anguillarum and *Aeromonas salmonicida* may cause lesions and ulcers in the skin as part of their pathogenesis (Brocklebank, 1998; Frans et al., 2011).

Moritella viscosa may cause large and deep penetrating ulcers. *M. viscosa* produces and excretes extracellular toxins also at temperatures as low as 4°C. This ability is probably one of the factors contributing to the observation that infections caused by *M. viscosa* often occurs at low temperatures, and that the disease is often called winter ulcers (Karlsen et al., 2014; Lunder et al., 1995). Historically the *M. viscosa* species has been divided into two genotypes, one typical, mostly affecting Atlantic salmon in Norway, Scotland and Faroe Islands, and another, atypical, mostly affecting rainbow trout, Atlantic salmon and lumpfish in Iceland and rainbow trout and cod in Norway (Grove et al., 2010). The bacterium is easily detected by cultivation at 2.5 % NaCl blood agar, it is β haemolytic and very viscous.

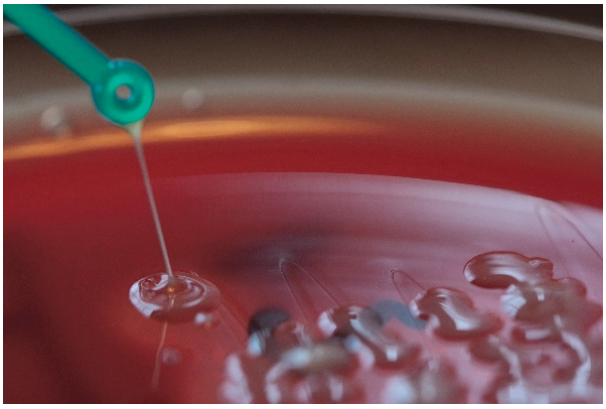

Figure 10. *Moritella viscosa* cultivated on blood agar (2.5 % NaCl) from ulcer in Atlantic salmon. Photo Øystein Klakegg

Aliivibrio wodanis is often isolated from fish with winter ulcers and from kidney from Atlantic salmon with winter ulcers, either alone or together with *M. viscosa (Lunder et al., 1995)*. *A. wodanis* can inhibit growth and virulence of *M. viscosa*, and the inhibitory effect is strengthened if *A. wodanis* colonized the fish before the exposure to *M. viscosa* (Karlsen et al., 2014).

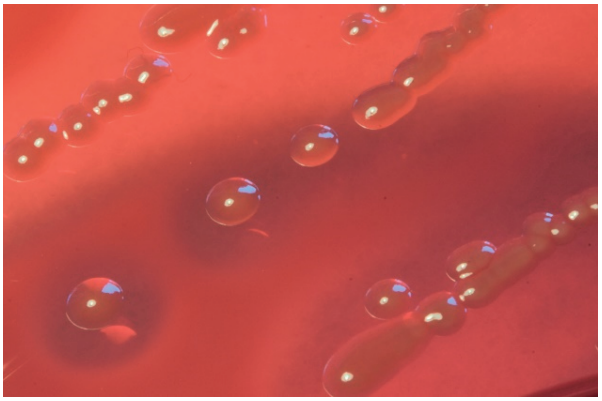

Figure 11. *Aliivibrio wodanis* cultivated on blood agar (2.5 % NaCl) from ulcer in Atlantic salmon. Photo Øystein Klakegg

Tenacibaculosis in fish was first described in 1977 (Masumura & Wakabayashi, 1977). In Atlantic salmon *Tenacibaculum maritimum*, *T. dicentrarchi* and recently *Tenacibaculum finnmarkense is* associated with disease and ulcers, and often with ulcers related to body and mouth (Avendano-Herrera et al., 2016; Avendano-Herrera et al., 2018; Avendano-Herrera et al., 2006; Småge et al., 2016). In Norway *Tenacibaculum* spp. have been isolated from Norwegian salmon since the 1980s, but it has often been unclear if the bacterium cause disease and also which *Tenacibaculum* sp. that has been detected (Olsen et al., 2011; Takle et al., 2015). *T.*

finnmarkense has been isolated from ulcers in farmed Norwegian Atlantic salmon particular in Northern Norway and infection trials have revealed that the bacterium causes disease (Småge et al., 2016).

Vibrio splendidus and *Aliivibrio logei* are often associated with ulcers from Atlantic salmon, but their importance is unknown (Benediktsdottir et al., 2015). It could be that these bacteria are opportunistic pathogens (Takle et al., 2015).

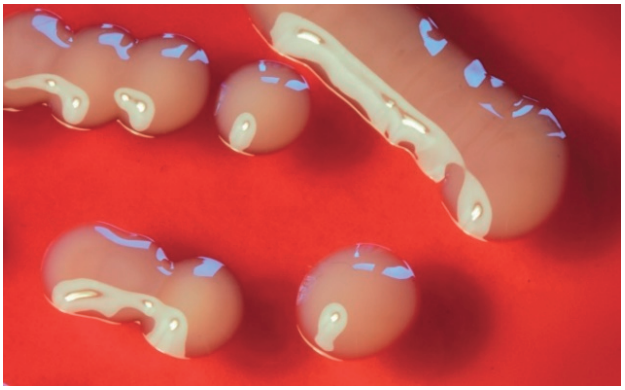

Figure 12. *Vibrio splendidus* cultivated on blood agar (2.5 % NaCl) from ulcer in Atlantic salmon.
Photo Øystein Klakegg

Flavobacterium psychrophilum, which mainly affects rainbow trout in freshwater in Norway, can cause ulcers in addition to sepsis in Atlantic salmon (Nematollahi et al., 2003).

Yersinia ruckeri can among other symptoms cause subcutaneous haemorrhages in the corners of the mouth in Atlantic salmon, mainly in freshwater, but lately more frequently also in sea water (Kumar et al., 2015).

Figure 13. From this diseased Atlantic salmon in sea water, *Y. ruckeri* was cultivated from lesions.
Photo Øystein Klakegg

There are effective vaccines protecting against *Vibrio anguillarum* and *Aeromonas salmonicida.* Most farmed Atlantic salmon are also vaccinated against *M. viscosa*, but winter ulcers still occur with some severity and frequency in association

with *M. viscosa* and other ulcerative bacteria. Vaccination against *A. wodanis* is not commercially used. In Iceland *A. wodanis* was used in vaccines for some years, but as the occurrence of winter ulcers was not reduced significantly, the vaccination against *A. wodanis* was terminated. Vaccine against tenacibaculosis has so far not been effective (Småge et al., 2018).

Lumpfish

In lumpfish *Moritella viscosa* is known to cause ulcers (Einarsdottir et al., 2018). *Pasteurella* spp., can also cause ulcers in lumpfish (Ellul et al., 2018).

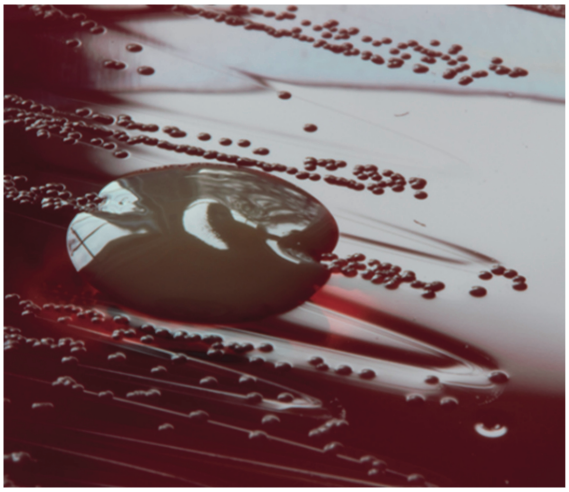

Figure 14. Pasteurella sp., probably *P. skyensis* cultivated on blood agar (2.5 % NaCl) from lesions from lumpfish, and one large *Vibrio* sp. colony. The presence of *Pasteurella* sp., inhibits growth of the *Vibrio* sp. as seen where the colonies of *Pasteurella* sp. are close to the colony of *Vibrio* sp. Photo Øystein Klakegg

Vibrio splendidus, *Aliivibrio wodanis* and possibly *Vibrio anguillarum* are also reported to cause skin lesions and rotten fins, particularly at the tail (Nilsen et al., 2014; Chakraborty et al., 2019). From crater disease, typified by circular ulcers less than 5 mm resembling a crater, *Tenacibaculum* sp. are frequently cultivated, but the relevance is not known (Pharmaq Analytic, 2019).

1.7 *Aliivibrio* spp. in Atlantic salmon and lumpfish

In 2007 four former *Vibrio* spp. were reclassified as *Aliivibrio* spp., namely *A. fischeri, A. logei, A. salmonicida* and *A. wodanis* (Urbanczyk et al., 2007*)*. They were reclassified due to the fact that the four *Aliivibrio* spp. were phenotypically distinct compared to other genera of the Vibrionaceae family and because their sequence identities for the 16S *rRNA* gene were higher than 97.4 % among the four members,

compared to less than 95.5 % sequence identity compared to the other nearest species to the Vibrionaceae, *Vibrio* spp. and *Photobacterium* spp. (Urbanczyk et al., 2007).

Phylogenetic analysis based on multiple independent loci and the 16S *rRNA* gene have revealed that substantial unrecognized diversity exists in the genus (Ast et al., 2009). Particularly strains that earlier were identified as *A. logei* now did not group with the type strain of *A. logei* (Ast et al., 2009). Three new species are proposed, *A. finisterre, A. sifiae* and *A. thorii,* and the indication was that there are more species in the genus (Ast et al., 2009).

Aliivibrio salmonicida has caused cold-water vibriosis, formerly called Hitra disease, in Norwegian salmon aquaculture since before 1979 (Egidius et al., 1986). The main clinical symptoms of the disease are anaemia and haemorrhages, particularly in the integument surrounding internal organs (Egidius et al., 1986). The first successful vaccination against the disease was reported in 1987 (Holm & Jørgensen, 2006). The bacterium seems to enter the fish via intact skin, and is detected in large numbers in blood only a few minutes after challenge (Kashulin & Sørum, 2014). After invasion to the tissues of the fish, *Al. salmonicida* (*Al*. to distinguish it from *Aeromonas salmonicida*) seems to avoid the host immune system, perhaps by muting gene expression (Bjelland et al., 2013). It could also be that the Toll-like receptors (TLR) of Atlantic salmon ignore the microbe-associated molecular patterns (MAMP) of *Al. salmonicida* as seen in some commensal bacterial species (Ausubel, 2005).

In a survey from April 2016, *Aliivibrio* spp. were cultivated from many ulcers and from two of nine head kidneys of Atlantic salmon with ulcerative disorders. The same seasonal peak was seen in fish intestines, where *Aliivibrio* were more abundant in April in fish suffering from winter-ulcer (Karlsen et al., 2017). Ribotypes related to both *A. logei* and *A. wodanis* were detected from the intestines (Karlsen et al., 2017).

A survey of bacterial contents from water and Atlantic salmon with proliferative gill inflammation using rt-PCR-DGGE (Denaturing Gradient Gel Electrophoresis) revealed *Aliivibrio logei*-like bacteria. Cultured samples from the same fish and sites revealed at least four species of *Vibrio/Aliivibrio* from the gill/seawater samples, *V. splendidus, V. ichthyoenteri, A. fischeri* and *A. wodanis*

(Steinum et al., 2016). The result that the cultured samples and the rt-PCR-DGGE identified different species, was probably because the 16S *rRNA* gene is very conserved for *Aliivibrio/Vibrio* genes that limits separation during gel-electrophoresis (Steinum et al., 2009).

In lumpfish, there are few investigations regarding *Aliivibrio* spp. In a survey in Norway in 2014, *Aliivibrio wodanis* and *Aliivibrio logei*-like bacteria were isolated from lumpfish at several sites. However, no conclusion was made as whether the *Aliivibrio* spp. were the cause of the problems and disease for the lumpfish (Hjeltnes et al., 2018; Nilsen et al., 2014).

1.8 *Tenacibaculum* spp. in Atlantic salmon and lumpfish

Bacteria in the genus *Tenacibaculum* are Gram-negative, yellow pigment producing thread-formed bacteria, 1.5-30 µm long. The genus *Tenacibaculum* is a member of the family Flavobacteriaceae. The species are isolated from the marine environment where they often are seen as "cleaners" since they have the ability to decompose large organic molecules such as cellulose and chitin (Suzuki et al.,2001). Tenacibaculosis in fish was first described in 1977 when *T. maritimum* was associated with disease in red sea bream (*Pagrus major*) and black sea bream (*Acanthopagrus schlegeli*) in Japan (Masumura & Wakabayashi, 1977).

Of the 31 species discovered so far in the genus, seven species are identified causing disease in fish: *T. maritimum, T. dicentrarchi, T. discolor, T. gallaicum, T. ovolyticum, T. soleae,* and *T. finnmarkense* (Fernandez-Alvarez & Santos, 2018; Lopez et al., 2010; Masumura & Wakabayashi, 1977; Pineiro-Vidal et al., 2012; Småge Brevik et al., 2016). The fish pathogenic species are often associated with ulcers related to the body and mouth, rotten fins, tail rot and necrosis and cornea ulcer (Avendano-Herrera et al., 2016; Avendano-Herrera et al., 2006; Pineiro-Vidal et al., 2012; Småge, Brevik et al., 2016; Småge et al, 2016). Some of the pathogenesis is probably related to the ability of these bacteria to degrade and take advantage in collagen and chitin (Takle et al., 2015).

In Atlantic salmon, *T. maritimum*, *T. dicentrarchi* and *T. finnmarkense* are all known to cause disease (Avendano-Herrera et al., 2016; Avendano-Herrera et al., 2006; Powell et al., 2004; Småge, Brevik, et al., 2016; Van Gelderen et al., 2010; van Gelderen, Carson, & Nowak, 2010, 2011).

In Norway the pathogenic role of the different *Tenacibaculum* spp. is still somewhat uncertain. *Tenacibaculum* spp. have been isolated from salmon and trout since the 1980s (Olsen et al., 2011). In 1995-96 *Tenacibaculum* bacteria were found in all 18 sites of Atlantic salmon investigated related to winter ulcer and in 70 % of the ulcers (Takle et al., 2015). In Norway, infections with *Tenacibaculum* spp. in Atlantic salmon have rarely been identified from the head kidney and have not been considered to be systemic. It has been uncertain whether the *Tenacibaculum* spp. isolated from the ulcers are the cause of the ulcers or just a secondary or opportunistic bacterium (Olsen et al., 2011).

T. finnmarkense was found to cause disease in Atlantic salmon in Norway (Småge, Brevik, et al., 2016). In addition, *Tenacibaculum* spp., phylogenetically close to *T. dicentrarchi* were isolated from four Atlantic salmon individuals in Norway, but the relevance for disease development was unclear since three of the four salmons were asymptomatic (Olsen et al., 2017). The same survey found uncharacterized *Tenacibaculum* isolates phylogenetically close to *T. dicentrarchi* from Norwegian Atlantic salmon and also found uncharacterized *Tenacibaculum* isolates clustered with *T. dicentrarchi* from diseased lumpfish in Norway (Olsen et al., 2017).

T. maritimum has been isolated from diseased farmed lumpfish with necrotic lesions in the skin in Norway (Småge et al., 2016). From lumpfish with "crater disease", *Tenacibaculum* sp. are frequently cultivated, but the clinical relevance of the bacterium remains unknown (Pharmaq Analytic). From biofilm, water and lumpfish eggs from some lumpfish farms with no disease outbreak, high relative abundance of *Tenacibaculum* spp. has also been detected (Roalkvam et al., 2019).

Most *Tenacibaculum* spp. do not grow on the most common agar media identifying fish pathogens as blood agar with 2.5% NaCl (Takle et al., 2015). *Tenacibaculum* spp. can grow on nutrient-poor medium containing seawater, and the most common medium is Marine agar (Olsen et al., 2011; Suzuki et al., 2001; Takle et al., 2015). *Tenacibaculum* strains grow slowly on other media and are often overgrown by other bacteria (Fernandez-Alvarez & Santos, 2018). These are some of the reasons why it is often difficult to cultivate or isolate *Tenacibaculum* species from fish tissues suspected to be infected with *Tenacibaculum* and consequently, tenacibaculosis is likely underdiagnosed (Olsen et al., 2011).

1.9 Indications that bacteria could be present within many tissues

Body tissues in eukaryotes have been considered as more or less sterile. It has also been assumed that healthy tissues beneath the epithelium lining the skin, the respiratory tract, the digestive tract and the urogenital tract are free of bacteria and that the epithelium of the skin or mucosa is an effective barrier preventing bacteria to get into or to be established underneath (Gaudier et al., 2006; Koziel et al., 2013).

Because of the general acceptance that healthy mesodermal tissues were sterile, the microbiome has mainly been investigated from gut, mouth, urogenital tract, skin, airways, breast milk or eye (Human Microbiome Project Consortium, 2012; Nelson et. al., 2013; Lloyd-Price et al. 2016).

Still, the finding of bacterial DNA in some healthy tissues, such as in lungs, prostate and synovial fluid has indicated that there could be bacteria also in healthy muscle, connective tissue and other tissues (Cavarretta et al., 2016; Pezzulo et al., 2013; Bhandal et.al., 2012). The role of the microbiome in non-mucosal cancers is under investigation, and there are indications that commensal bacteria both affect inflammation in the sterile tumor microenvironment and that optimal responses to cancer treatment require an intact commensal microbiota in some cases and that malign tumors have regressed following infections (Moran and Al-Hebshi, 2020; Lida et al., 2013; Kapp, 1983). The presence of bacteria within tumors could be due to infection via the vasculature. The bacteria are thought to gain access to the systemic circulation from the gut through the enteric venous system to the portal vein or via the lymphatic drainage. The lymphatic route is thought to be the main pathway of bacterial translocation from the gut mucosa into normal sterile tissue (Berg et al.,1979; Balzan et al., 2007; Cummins and Tangney 2013).

The existence of an *in utero* colonization of the mammalian fetus has also been subject of investigations. The evidence of the microbiota presence in placental and fetus tissues is not consistent. Theis et al. isolated a common skin bacterium, *Streptococcus hominis*, from the fetal intestinal tissue sample from mice (Theis et al., 2020). Other studies including culture independent techniques have found bacterial DNA in placenta, amniotic fluid, meconium and fetal intestines (Martinez et al., 2018; Funkhouser and Bordenstein, 2013; Jimenez et al., 2008; Aasgaard et al., 2014, Collado et.al., 2016; DiGiulio et al., 2008). Investigations of intra-amniotic infections

in humans suggests that the ascending pathway from the lower genital tract is the most common pathway, but that also hematogenous dissemination from the oral cavity or intestinal could be a potential pathway (Romero et. al., 2019).

Although part of the identification of bacteria in tissues other than the epithelium is done by cultivation, the new molecular-based cultivation-free methods of identification of microbial populations have made identification easier. Metagenomic studies are now performed routinely in many labs (Nelson et al., 2013). As only a small fraction of the bacteria is culturable, this can also partially explain why the whole microbiome has not been studied more in depth earlier (Amann et al., 1995; Zhou et al., 2014).

It is predominately in mammals such as humans where bacterial occurrences in non-epithelial tissues have been investigated, but some findings of prokaryotic rRNA and DNA in black spots in salmon fillets suggest there could be bacteria there as well (Krasnov et al., 2016).

1.10 **Knowledge gap**

With a growing population of humans, eating more protein, in a world that already is on the edge of sustainability, it is very important to secure, utilize and optimize the resources we put into the food we make in productive systems like aquaculture, for example: fish eggs, smolts, water and feed. A better feed utilization rate, increased survival, good animal welfare and less waste will all contribute to improved sustainability.

More than 15 % of Atlantic salmon put in sea water in Norway are lost (Norwegian Directorate of Fisheries', 2019). The economic feed conversion rates vary threefold between different companies and sites in Norway. These factors require finding new effective solutions to tackle the current situation in fish industry. The new solutions are equally vital for fish industries outside Norway because of the similarity in current statistics concerning economic losses due to fish infectious diseases (Adams, 2019).

Despite extensive use of vaccines against the bacterial diseases believed to cause the largest problems in salmon, there is still considerable mortality among farmed Atlantic salmon (Aunsmo, 2008; Hjeltnes et al, 2018; Norwegian, Directorate of Fisheries', 2019). Despite the use of vaccines against winter ulcers, there are still many fish that get ulcerative bacterial disease, and the vaccine against winter ulcers

do not protect very good (Aunsmo, 2008; Hjeltnes et al, 2018; Sommerset et al., 2021). The knowledge of which bacteria that are causing these ulcers is lacking or incomplete. It is not unlikely that there are a number of bacteria that contribute to the development of ulcers. Knowledge about these bacteria and their interactions is still inadequate. There is also little understanding why salmon on some occasions can be produced without ulcers when at other times at the same site with the same operations, the fish are observed with massive outbreaks of ulcers. We often find *Tenacibaculum*-like bacteria in ulcers in Atlantic salmon, but they are difficult to grow, and the knowledge if *Tenacibaculum* spp. cause ulcers and disease and what species of the genus *Tenacibaculum* that eventually cause the disease is largely unknown (Olsen et al., 2011). Description and verification of more bacteria causing disease in both Atlantic salmon and lumpfish will aid in the prevention of those bacterial diseases.

Despite our increasing understanding of the bacterial microbiota in fish, there is very much we do not know yet. The knowledge of how bacteria and host interact is also still inadequate. Our knowledge about the inter-bacterial interaction and interaction between bacteria and fish is largely missing. Gaining more insights into causation of fish infection diseases is required. Also, the research in the area of probiotic application in aquaculture is in the process of development. There are examples a direct inhibition of fish-associated bacteria by other distinct members of the marine biome. For instance, a pathogen, *Aliivibrio wodanis* inhibits another pathogen bacterium *Moritella viscosa* (Hjerde et al., 2015; Karlsen et al., 2014). This existing knowledge of microbe-microbe interaction motivates a search for beneficial fish-associated bacterial species that could enhance fish robustness against infectious disease and act in a probiotic like way. Previously, several novel *Aliivibrio* spp. (*A. njordis* NCIMB 42593 (VL1), *A. balderis* NCIMB 42592 (VL2), and *A. nannie* NCIMB 42594 (VL3)) with possible probiotic properties have been isolated from Atlantic salmon (Patent US, 2020). The ability of these *Aliivibrio* spp. to act as probiotics should be investigated.

Besides, probiotic supplementation via rearing water is not commonly used in aquaculture including Atlantic salmon rearing. To the best of our knowledge, this way of probiotic supplementation has never been used in lumpfish.

2. AIMS OF STUDY

The main goal of this project was to gain knowledge about possible probiotic effects of novel host-associated *Aliivibrio* spp. on Atlantic salmon and lumpfish as estimated by growth, feed utilization, health, and resistance against pathogens.

Sub objectives:

- Identify and genetically characterize bacteria that were involved in an outbreak characterized by ulceration, fin rotting, and an increased mortality in Atlantic salmon in Norway. To confirm pathogenicity potential of the identified strain using an infection model.

- Supply one, two or three *Aliivibrio* spp. with probable probiotic effect to Atlantic salmon and lumpfish and measure effects on feed utilization, growth, ulcers and survival.

- Investigate if potentially probiotic *Aliivibrio* spp. affect the effect of potentially pathogenic bacteria as *Moritella viscosa*.

3. SUMMARY OF PAPERS

Paper I:

An outbreak of acute disease and mortality in Atlantic salmon (*Salmo salar*) post-smolts in Norway caused by *Tenacibaculum dicentrarchi*

Øystein Klakegg, Takele Abayneh, Aud Kari Fauske, Michael Fülberth, Henning Sørum

An outbreak of disease characterized by skin ulcers, fin rot and mortality was observed a few days after the transfer of Atlantic salmon (*Salmo salar*) from a freshwater smolt production facility to a land-based seawater post-smolt site.

Dead and moribund fish had severe skin and muscle ulcers, often 2-6 cm wide, particularly caudal to the pectoral fins.

Microscopic examination of smears from ulcers and head kidney identified long slender Gram-negative rods.

Histopathological analysis revealed abundance of long slender *Tenacibaculum*-like bacteria in ulcers and affected fins.

Genetic characterization using Multi-locus Sequence Analysis (MLSA) of seven housekeeping genes including *atpA, dnaK, glyA, gyrB, infB, rlmN* and *tgt*, revealed that the isolates obtained during the outbreak were all clustered with the *Tenacibaculum dicentrarchi* type strain (USC39/09^{T}) from Spain. Two bath challenge experiments with Atlantic salmon and an isolate of *T. dicentrarchi* from the outbreak were performed. No disease or mortality was observed in the first trial. In the second trial with a higher challenge dose of *T. dicentrarchi* and longer challenge time, 100 % mortality occurred within 48hr.

This is the first reported outbreak of disease caused by *T. dicentrarchi* in Norwegian farmed Atlantic salmon.

Paper II:

Enhanced growth and decreased mortality in Atlantic salmon (*Salmo salar*) after probiotic bath

Øystein Klakegg, Kira Salonius, Arve Nilsen, Michael Fülberth, Henning Sørum

Outbreaks of disease in farmed Atlantic salmon occur in all its life stages. Salmon are particularly vulnerable to infectious diseases at transition from the

freshwater stage to the saltwater stage. To reduce the mortality and improve the health of farmed Atlantic salmon, microbial enhancement with a probiotic bath or a dip approach has been tested.

In three trials at two sites in Norway, one, two or three probiotic isolates of *Aliivibrio* bacteria were added to the rearing water. The Atlantic salmon were bathed in the probiotic bacteria for a period from 30 to 60 minutes. The time of bathing was at transfer to sea water or 57 days before transfer to sea water. The salmon were from 55 gram to 110 gram in size at the time of bathing in the different trials. The fish were followed in four to six months after bathing with observations and samplings. Growth, ulcers and survival were recorded. Feed conversion rates were recorded in trial 1 and 2.

The growth was better for the probiotic groups. In trial 1, fish in the probiotic enhanced group were 31 % heavier at end of trial compared to the weight of the fish in the control group and in trial 2 the probiotic enhanced groups were from 11 to 25 % heavier. The feed conversion rate in trial 1 and 2 was 9-28 percent lower and significantly better for the probiotic enhanced group. In trial 3 a natural outbreak of disease caused by *Moritella viscosa* occurred, and the fish bathed in the highest concentration of the probiotic bacteria had significantly lower mortality than in the control groups.

Bathing or dipping of Atlantic salmon with probiotic *Aliivibrio* strains increased growth, reduced mortality and improved FCR.

Paper III:

Improved health and better survival of farmed lumpfish (*Cyclopterus lumpus*) aftera probiotic bath with two probiotic strains of *Aliivibrio*.

Øystein Klakegg, Siri Myhren, Rosemary Alice Juell, Marit Aase. Kira Salonius, Henning Sørum

Cleaner wrasse eats sea lice (*Lepeophtheirus salmonis*) directly from the skin of the Atlantic salmon (*Salmo salar*). To reduce harvest from the wild wrasse populations and to increase the quality and availability of cleaner fish, lumpfish (*Cyclopterus lumpus*) farming has been increasingly developed as an industry to service the farming of Atlantic salmon in the last few years. Acquired resistance against the de-lousing chemicals at an increasing speed particularly in mid- and

western-Norway has made the farming of lumpfish more important in the control of the sea lice infestations in farmed Atlantic salmon.

Outbreaks of disease in farmed lumpfish occur in all its life stages. Some outbreaks of disease caused by pathogenic bacteria are resulting in high mortality while other bacteria cause lower but more chronic mortality. To reduce the mortality and improve the health of farmed lumpfish microbial enhancement with a probiotic bath approach has been tested.

In five trials at two lumpfish aquaculture facilities in Norway, two probiotic isolates of *Aliivibrio* bacteria were added to the rearing water. The lumpfish were bathed in the probiotic bacteria in 10-30 minutes, the cfu (colony forming units) of probiotic bacteria in the rearing water varied from 7.5 X 10^5 to 5 X 10^7per ml. The average weight of the lumpfish at the time of bathing were from 0.025 g to 16.3 g in the various trials. The lumpfish were observed and sampled from 45 to 87 days after bathing. Growth, ulcers and survival were recorded. The survival of lumpfish in trials 3, 4 and 5 in which the fish were bathed 1 to 2 weeks before vaccination was significant better for the probiotic exposed groups compared to the control groups. In trials 4 and 5, a natural outbreak of disease caused by *Moritella viscosa* occurred. The groups exposed to probiotic bacteria had significant fewer ulcers than the control groups.

The growth of the fish exposed to probiotic bacteria at weight 0.025 g was significant better in the bathed group compared to the controls. In three trials the growth of the probiotic exposed lumpfish one to two weeks before vaccination was also higher than of non-exposed fish.

Bathing of lumpfish with two *Aliivibrio* bacteria increased growth and made the fish more resistant to bacterial diseases. To our knowledge, this is the first scientific report of probiotic bacteria applied to lumpfish.

4. RESULTS AND GENERAL DISCUSSION

Bacteria communicate and interact both with other bacteria and with the eukaryotic host, and many bacteria are important for a well-functioning eukaryotic organism. A bacterium is not only a potential pathogen, but in many cases essential for a healthy life and eukaryotes and bacteria are often living in synergy (Bang et al., 2018; Esser et al., 2018; Ezenwa et al., 2012; Morris, 2018).

Losses and mortality in aquaculture are common worldwide. In Norwegian salmon farming about 15 % of the fish put to sea is lost. This includes dead fish, downgrading at slaughterhouses and escapees. Dead fish is the largest contributor to losses (Norwegian Directorate of Fisheries, 2019). Ulcers and fin lesions contribute much to both mortality and downgrading.

The challenges with sea lice infestation that affect the skin integrity and the increasing resistance against chemicals used for many years in the control of sea lice have resulted in the introduction of alternative mechanical and thermal methods to keep the level of sea lice below the permitted number per fish. The salmon farmers in Norway need to perform a mandatory reduction of the level of sea lice when the average number of sexually mature sea lice per salmon passes 0.5 or 0,2 (when wild smolt are migrating) which is the upper limits of sea lice permitted by the Norwegian National Health Authorities. The reason for keeping such a low level of sea lice in farmed salmon is the threat against the wild salmon smolt when they leave the rivers for the ocean feeding in the North Atlantic ridge north and south of Iceland. The low level of sea lice before a mandatory reduction of the number of sea lice has left the farmed fish almost without damages from the sea lice activity in their skin but farmed salmon are stressed to a large extent by repetitive de-lousing activities with mechanical and thermal methods. During the warm seasons net pens in many areas are de-loused more than ten times to keep the number of sea lice below the limit given by the authorities, and the average numbers of de-lousing treatment in some areas are more than 6 times per year (Kyst.no, 2020) and this have increased the importance of a healthy and strong fish skin further.

The introduction of cleaner fish, as lumpfish, to the cages with farmed Atlantic salmon to control sea lice infestations should make us look at the salmon and cleaner fish more closely together. In lumpfish the losses, mostly due to

mortality, are even larger than for salmon, probably more than 50 % of the individuals are lost. Ulcers contribute to mortality also in lumpfish, and the sustainability and animal welfare regarding use of cleaner fish as lumpfish is debated. The Norwegian Food Safety Authority wrote the following statement in its cleaner fish campaign report in 2019 "The Aquaculture farmers cannot continue to use cleaner fish in large scale unless the conditions for cleaner fish improve significantly" (Mattilsynet, 2019). Therefore, the knowledge about skin ulceration and fin lesion pathogenesis and, specifically, the role of bacterial causative agents in Atlantic salmon and lumpfish is required. How bacteria, including probiotic bacteria in collaboration with each other affect and interact with both Atlantic salmon and lumpfish is understood to only a minor extent. The bacterial populations of lumpfish and Atlantic salmon have partial qualitative similarities, i.e some bacterial species are shared between the two species (paper 3; Burtseva et al. 2021). It is probable that the lice-eating lumpfish may transmit pathogens to the fish they are intended to help or vice versa as we have seen with *Pasteurella skyensis* (Veterinærinstituttet, 2019). Similarly, probiotic good bacteria in one species can be transmitted to other close living species.

Currently, much of the mortality and morbidity in both Atlantic salmon and lumpfish are due to ulcers and bacterial diseases (Hjeltnes et.al. 2018). Despite vaccination against some bacterial infections causing ulcers, bacteria cause major problems with ulcers in both Atlantic salmon and lumpfish. In 2019, the National Norwegian Veterinary Institute recognised that ulcer caused by bacteria as an increasing problem in Norwegian aquaculture (Veterinærinstituttet, 2020). There is a need to identify to be able to prepare for better mitigation and prevention of these infections. There are also probably species of pathogenic bacteria not included in the vaccines applied in the Norwegian aquaculture causing infections and losses. The effect of vaccines against ulcers are particularly low, and 0 (zero) percent of the fish health employee responding to a survey regarding protection after vaccination of Atlantic salmon against ulcers (*M. viscosa*) in Norway in 2020 respond that the protection against ulcer after vaccination was adequate (Sommerset et al., 2021). Against vibriosis or furunculosis 80 percent respond that the protection was adequate. It was therefore interesting to investigate the cause of ulcer and disease when skin ulcers, fin rot and mortality was observed a few days after the transfer of

smolts from a freshwater smolt production facility to a land-based seawater post-smolt site in a Norwegian salmon farm that were involved in a research project related to ulcer surveillance. The total number of Atlantic salmon in the group was 611 000. Dead and moribund fish had severe skin and muscle ulcers, particularly caudal to the pectoral fins (Figure 15).

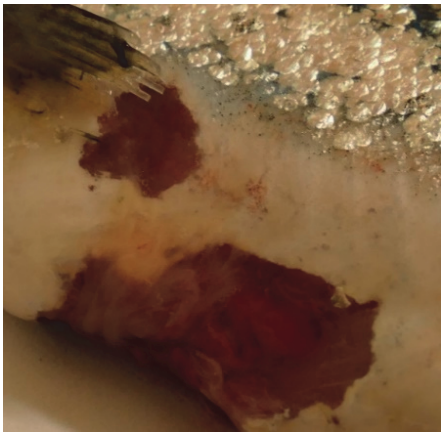

Figure 15. Moribund fish with typical ulcer caudal to the pectoral fin and an ulcer perforating the abdomen.
Photo Øystein Klakegg

Microscopic examination of smears from ulcers and head kidney and histopathological analysis revealed a high number of long slender *Tenacibaculum*-like bacteria in ulcers and affected fins.

Genetic characterization using Multi-locus Sequence Analysis (MLSA) of seven housekeeping genes including *atpA, dnaK, glyA, gyrB, infB, rlmN* and *tgt*, revealed that the isolates obtained during the outbreak were all clustered with the *T. dicentrarchi* type strain (USC39/09^{T}) isolated from European sea bass (*Dicentrarchus labrax*) in Spain (Figure 16). The MLSA also revealed that the outbreak of disease was caused by different allele types of *T. dicentrarchi*, and therefore that the water probably carried the primary source of *T. dicentrarchi* in the outbreak and not one or a few diseased fish (paper 1).
In the second challenge trial, we got 100 % mortality within 48 hours after challenge. Blood samples from moribund fish, revealed a sepsis with lots of *T. dicentrarchi* in the blood.

In Norway this is the first reported outbreak of *T. dicentrarchi* in Atlantic salmon.

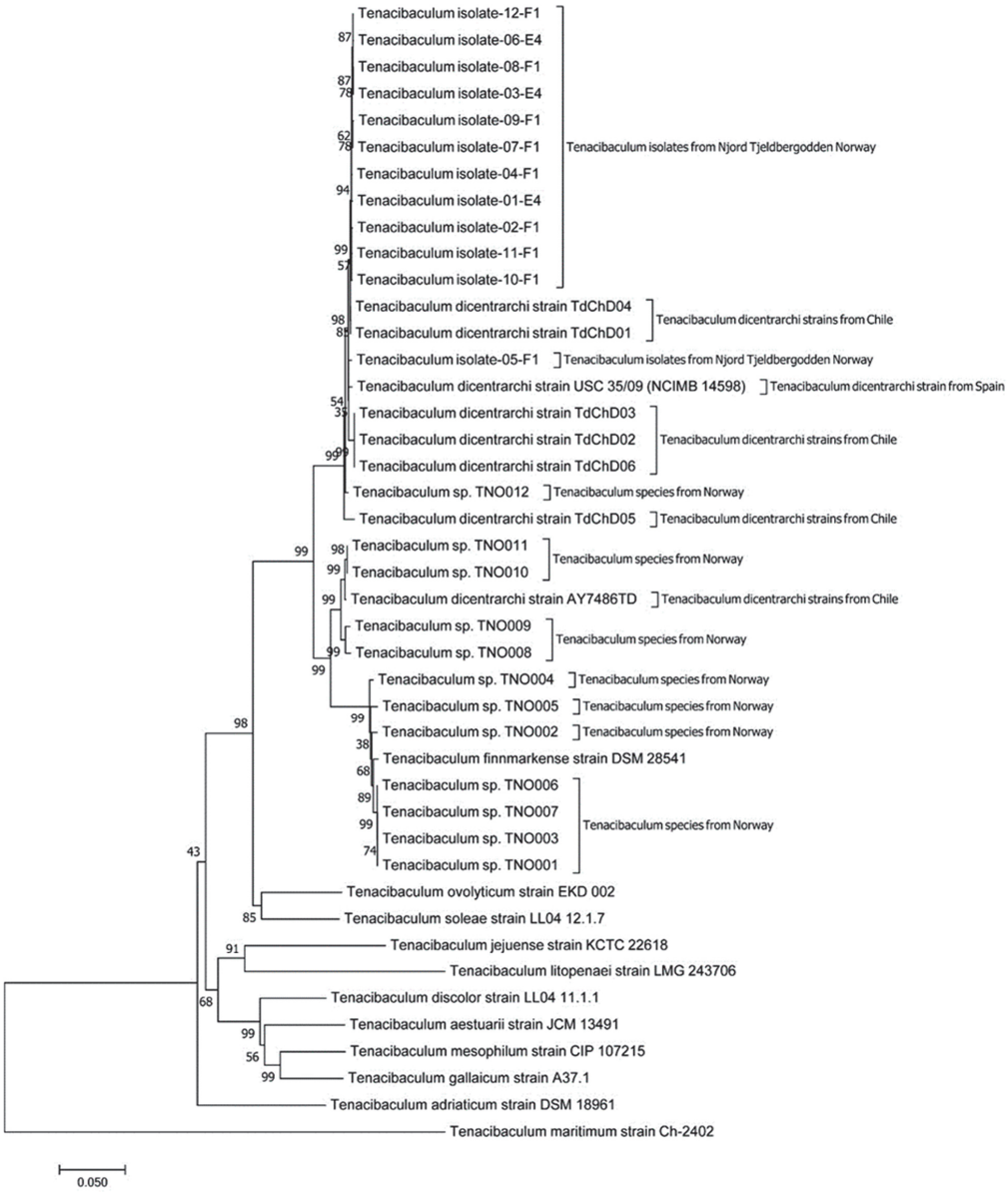

Figure 16. Multi-locus Sequence Analysis (MLSA) of the seven housekeeping genes: *atpA, dnaK, glyA, gyrB, infB, rlmN* and *tgt*, revealed that the isolates obtained during the outbreak (isolates 1-12) were clustered with the *T. dicentrarchi* type strain. Slightly modified after Figure 7, paper 1.

Regarding ulcer, disease and mortality in fish some ulcers appear suddenly in the populations and affect many fish at the same time and have an acute expression while other ulcers, diseases and mortality appear more slowly, affect a smaller number of fish at the same time and have a more chronic expression. The outbreak of disease caused by *Tenacibaculum dicentrarchi* described in paper 1 appeared abruptly with fin rot ulcer and mortality within a short time period. There was also a second rise in mortality five to seven days after the first onset of mortality, indicating a new rise in the concentration of *T. dicentrarchi* in the water shedded from the first group of diseased salmon that infected both uninfected salmon and intensified the infection of the already infected, but not yet immunized, salmon.

The outbreak of acute disease and mortality caused by *T. dicentrarchi,* described in paper 1, contrasts with many of the more chronic diseases with slow developing course, usually moderate daily mortality, but often high cumulative mortality over time. Disease and mortality often tend to increase after stressful operations and during periods of poor water quality indicating hypoxic seawater is a risk factor for disease (Jones et. al., 2020, Korkea-aho et. al., 2008). Increased mortality after hypoxia could partly be explained by depressed immune response, although there are indications that moderate hypoxia does not negatively affect the innate immune response in Atlantic salmon (Zanuzzo et al., 2020).

There may be difficult to provoke disease in challenge trials with pathogenic microorganisms. *T. dicentrarchi* was isolated from both diseased commercially farmed Atlantic salmon and lumpfish. *T. dicentrarchi* were further detected in cages with high, acute mortality and then observed in massive numbers from both microscopy of smears, as well as through cultivation from and by histology of affected tissues, mostly in pure occurrence. Challenge studies performed to fulfil Koch's postulates do not cause disease every time as the first *T. dicentrarchi* challenge to Atlantic salmon, described in paper 1. It is often difficult to cause diseases in bacterial challenge studies, not least with tenacibaculosis (Baxa et al., 1987; Lunder et al., 1995; Masumura & Wakabayashi, 1977; Powell et al., 2004). An acute outbreak of disease was observed in the second of the *T. dicentrarchi* challenge trials. We do not know why the first *T. dicentrarchi* challenge trial did not trigger visible disease, but it could be that fish were more resilient against the potential pathogenic bacteria, because commensal bacteria in that population helped to

protect the fish. One of the factors considered regarding disease causation is that commensal bacteria in some cases protect the host against pathogenic bacteria (Byrd & Segre, 2016). It could also be other factors such as different immune status, different concentration of infective bacterial cells among others. Challenge trials with multi-factorial diseases are often difficult. Sometimes we get disease as intended, and other times we do not. There were many parameters that we did not have under control or they were different from the disease we observed at the aquaculture sites and in the challenge trials, among others: the genetic make-up and the immune response of the fish, the virulence of the bacteria – maybe changed after multiple cultivations, the existence of other bacteria in the rearing water or fish depressing or activating the pathogen and differences in water temperature. Both the stress level of the fish, exposure time to the pathogen, concentration of the bacteria, the type and occurrence of pre-existing lesions in the fish may differ. We got disease and mortality in the second challenge trial with *T. dicentrarchi*. The concentration of bacteria in the water was $4X10^7$ cfu/ml, a challenge dose that in most cases probably are much larger than what we got in commercial fish farming. It could therefore be questioned how relevant, regarding commercial fish farming, challenge studies with huge doses of bacteria are.

Chronic diseases are probably often worse to define and diagnose and they are perhaps also more profoundly influenced by factors other than the pathogen itself, and this to a larger extent compared to more acute diseases. In multi-factorial scenario, typical of many diseases, including infectious ones, we sometimes see a manifestation of disease and other times not. There is emerging evidence that some diseases in marine systems are caused by microbial imbalance or dysbiosis with shift in microbial community structures (Egan & Gardiner, 2016). One example is the increased abundance of *Vibrio* spp. within Atlantic salmon displaying clinical signs of yellow mouth disease caused by *T. maritimum* (Wynne et al., 2020). As long as we do not have the complete knowledge, there may be apparent coincidences that somewhat occasionally account for disease. Koch's postulates was developed to describe acute infections caused by a single pathogenic agent, and thus have limitation for multi-factorial and chronic diseases (Ross & Woodward, 2016). Challenge trials are challenging.

We know that probiotic bacteria in some cases reduce the impact of bacterial diseases. As mentioned in paper 2, the rationale for the first trial of bathing Atlantic salmon with the potential probiotic *Aliivibrio* spp. was to find a solution to reduce the repeated occurrence of ulcers and mortality in the post smolt facility within the various groups of post smolt introduced to the facility. The actual facility was the same as the one in which the outbreak caused by *T. dicentrarchi* happened.

Three trials at two different sites, bathing Atlantic salmon with *Aliivibrio* spp. (*A. njordis* NCIMB 42593 (VL1), *A. balderis* NCIMB 42592 (VL2) and *A. nannie* NCIMB 42594 (VL3)) are described in Paper 2. The main purpose of this study was to evaluate if adding one, two or three of the potential probiotic *Aliivibrio* spp. into the rearing water, would positively affect the growth, health, feed conversion rate and survival of Atlantic salmon. In addition, we wanted to determine if there is a concentration dependency for the probiotic effect of the bacteria added to the rearing water of salmon. We also wanted to get an indication of whether there is any difference in the probiotic effect by applying single strains or different strain mixtures of the three potential probiotic strains used in this study.

These trials included from 800 to 16 000 fish and revealed better growth, less ulcers and less mortality in the bathed groups compared to the controls. In trial 1, the results demonstrated 47 percent fewer post smolt salmon with ulcers, 31 percent increased weight of the post smolts and a feed conversion rate that was 28 percent better in the group bathed in *Aliivibrio* spp. versus the control group. In one of these trials in paper 2, where we were looking for concentration dependency, the salmon were impacted by a natural outbreak of disease caused by *Moritella viscosa*. The mortality was significantly lower ($p<0.05$) in the group bathed in the highest concentration (1/100) of probiotic bacteria compared to the control, and interestingly the antibody response against *M. viscosa* tended to be inversely proportional to the concentration of bacteria in the probiotic bath. The difference regarding antibody response was not significant at level $p< 0,05$.

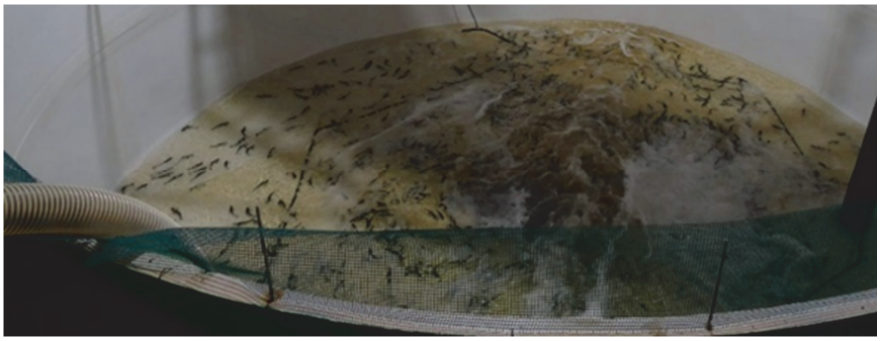

Figure 17. Bathing of Atlantic salmon with probiotic *Aliivibrio* spp. Photo Øystein Klakegg

As mentioned in paper 3, some of the potential probiotic *Aliivibrio* spp. used in the trials described in paper 2 and 3 are also found in lumpfish. And as mentioned in paper 3, the cultivation of bacteria from lumpfish, both sick and healthy, revealed that pathogenic bacteria and the microbiota of lumpfish were overlapping with what we find from other fish living in the same marine environment. The same probiotic *Aliivibrio* spp. that have been found and used with Atlantic salmon as probiotic bacteria were also found in lumpfish, particularly in large and healthy fish. Therefore, to investigate if exposing lumpfish to potential probiotic *Aliivibrio* spp. could reduce mortality, increase growth and resistance against bacterial diseases, lumpfish were bathed with *A. njordis* NCIMB 42593 (VL1) and *A. balderis* NCIMB 42592 (VL2) to investigate possible probiotic effects of these strains in lumpfish (Paper 3). The land-based facility where 4 of the 5 trials with lumpfish were done was located next to, about 100 meters from, the land based site where the outbreak of disease in post smolt salmon caused by *T. dicentrarchi* described in paper 1, happened. The water intakes for both facilities were located in the same area. We also cultivated *T. dicentrarchi* from the lumpfish at this location (data not shown).

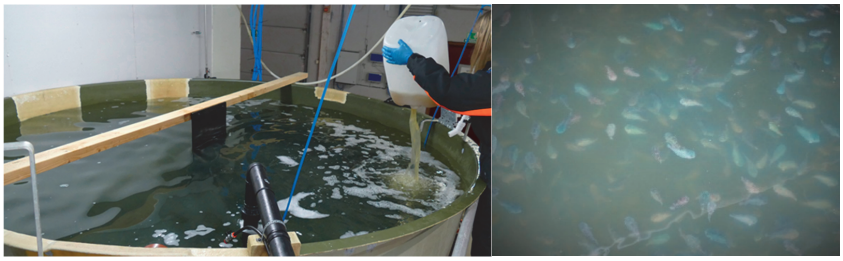

Figure 18. Application of probiotic bacteria into a tank with lumpfish.
Photos Øystein Klakegg

Figure 19. Some of the lumpfish that bacteria were collected from.

Photos Øystein Klakegg

The effect of bathing lumpfish in the potential probiotic bacteria was positive, especially when bathing fish more than 5 grams in size prior to stressful operations and challenges, such as vaccination. Overall, the growth was larger in three of the four trials where lumpfish were bathed in a bacterial concentration of >1 $X10^7$ cfu per ml of water, the mortality was lower, and, to a certain extent, the probiotic bacteria protected against natural outbreaks of disease caused by *Moritella viscosa*. See paper 3.

Two of the three trials with Atlantic salmon and all the trials with lumpfish were performed at commercial aquaculture sites. Performing trials at commercial sites has often some large advantages. The numbers of fish in each trial are often large, diminishing the random differences in each individual, and the conditions for the fish are close to the average of the industry, so interventions and procedures performed in such trials will be easier to implement in the industry. In our trials we could follow many more fish than we could do in a research site with smaller populations due to both economy and tank size. The fish sampled bacteria from and bathed with probiotic bacteria from the commercial aquaculture facilities were treated similarly as the other fish at those sites regarding vaccinations, feeding and husbandry.

A disadvantage when doing trials at commercial sites is that the experimental setup seldom is optimal. We should have had more tank replications, more equal numbers of fish in each tank, several more measurements and weighings of fish etc, that is mostly not possible in commercial facilities. Another clear disadvantage when performing field trials is that the trials almost never run as designed. The commercial sites often change plans due to customers, space, unforeseen things, accidents and so on. Some of the experiments we performed are not mentioned in the scientific reports since unforeseen and not planned events occurred, for instance that bathed groups and control groups were mixed.

One of the challenges we dealt with was to sample fish representatively, since we could not, due to large numbers of fish, sample all the fish each time. When sampling Atlantic salmon, a large circular (diameter 100 cm) net was lowered to the bottom of the tank and pulled up as quickly as possible by hand after about one minute for weighing from the large tanks (150 m^3 and 835 m^3) in the commercial aquaculture site to follow the smolts bathed with probiotic bacteria (Figure 20). The

impression was that the fish we sampled were somewhat smaller than the average size of fish in the tanks, and that the largest fish escaped from the net when we were pulling the net upwards. Other methods were discussed. To weigh all fish was too time-consuming and intrusive. Quick samplings with small handhold nets were considered to give even more skewed samplings. The same methods were used in all tanks, so the relative weights between the different tanks should be comparable. It was the relative growth between the groups that was most interesting compared to results with the absolute weight in each tank.

Figure 20. Sampling fish from a 150m^3 tank. Photo Øystein Klakegg

When sampling lumpfish for body weight we used a handheld net. Lumpfish from the same hatching and age differed much in weight, much more than in the case of Atlantic salmon. The observation was also that lumpfish of same size tended to stay together. It was therefore very important to take the samplings from many different and representative places in the tank. When sampling, it was the same person that did all the catching on a particular day with a net for every tank to make the samplings as equal as possible from tank to tank.

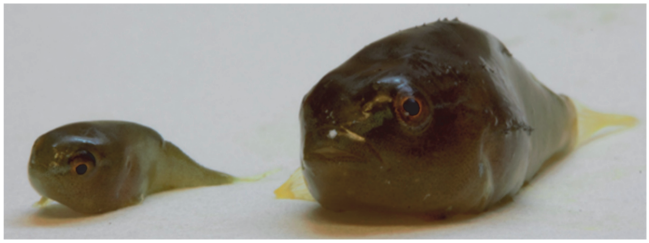

Figure 21. Lumpfish from the same hatching often differed considerably in weight. Photo Øystein Klakegg

The existence of too few tanks and lack of tank replication both regarding exposed groups and non-exposed groups (controls) was another challenge we dealt with. This may have confounded the results by the tank effects. Ideally, we should

have had tank replicates of all different applications and controls in each trial, preferably triplicates, to diminish the likelihood of a possible tank effect. Regarding microbiome, there is also a tank effect and it is known that the microbiota of a tank and the fish in the tank can be compositionally close and that there is a strong association between tank and the microbiome (Minich, 2020). We had, unfortunately, to deal with the number of tanks and the number and size of fish in each tank that the commercial sites made available to us. In trial 2, 4, 5 and particularly 3 in the trials described in paper 3 the number of fish in the control group(s) and the bacterial enhanced groups varied considerably. Different number and density of fish in each tank could also affect the trials and should preferably have been avoided. The number of fish in the control groups were higher than the number of bacterial enhanced fish in three trials and smaller in one trial.

The tank effect on growth and other parameters has been previously shown (Speare D. et al., 1995). To diminish the risk of tank effect, the control tanks in trials 1 and 2 in paper 2 were different in the two trials. The trials in paper 3 was performed in different tanks each time. In trial 4 in paper 3, both the treated group and the control group were split into two tanks one week after start of trial, reducing the possible tank effect. In trial 3 described in paper 2, we had two control tanks, but only one single tank of each concentration of specific probiotic bacteria. Since the two pairs of bacteria *A. njordis* and *A. balderis*, and *A. balderis* and *A. nannie*, were considered to be fairly similar and with positive effects compared to the controls, they were considered as almost duplicates when planning the trial. To reduce the tank effect in both papers 2 and 3 ideally, we should have had several tanks, both treated and controls, and the same number of fish with equal weight in each tank. A better setup in trial 3 paper 2 would probably have been to only test one combination of probiotic bacteria and then in duplicates or triplicates.

Feeding of fish and to have control over the amount of feed given to the fish was another challenge we dealt with at the commercial sites. In trial 1 and 2 in paper 2 all the feed was weighed, and the fish were mainly fed a daily ration according to its total biomass and adjusted for appetite, and overfeeding was attempted to be avoided. In trial 3 in paper 2 the feed was not weighed but given according to appetite. In all the trials described in paper 3 the amount of feed was adjusted to appetite, with some degree of overfeeding. When the feed is adjusted for appetite,

the subjective assessment done by the person adjusting the amount of feed is very important. When overfeeding, a small difference in assessment is not that important. When not overfeeding, the assessment is more important regarding growth because the likelihood that some fish, due to hierarchy, do not eat all the feed that they want or need is larger. To reduce the risks for different feeding between the tanks, the same person assesses the daily ration of feed in all the tanks in the same trials the same day. Ideally, we should have weighed all feed we put into the tanks, have a little overfeeding, and weighed the feed in the outlet water. Unfortunately, this was not possible.

Since the experimental settings were not optimal, despite large groups, we must interpret the results with cautions. The growth was significantly ($p<0,05$) better in all the four bacterial enforced tanks compared to the respective control tanks in trial 1 and 2 involving Atlantic salmon and in three of four trials (trial 1, 3 and 5) in which lumpfish were bacterial enforced with more than $1X10^7$ cfu *Aliivibrio* spp./ml. The mortality rate was also significantly lower ($p<0,05$), compared to the control groups, in the two trials with Atlantic salmon bathed with more than $1X10^7$ cfu/ml *Aliivibrio* spp. in which there was some mortality (trial 1 and 3) and in all the three trials where lumpfish were bathed with more than $2X10^7$ cfu/ml *Aliivibrio* spp. 1-2 weeks prior to vaccinations ($p<0,01$). This is also an indication that these interventions made a positive effect to both Atlantic salmon and lumpfish regarding mortality. The improved feed conversion rate (fcr) in the two trials (trial 1 and 2, paper 2) with Atlantic salmon in which the fcr was calculated and the improved growth observed in most of the trials are also indications that bathing Atlantic salmon with the *Aliivibrio* spp. is improving the feed utilization. We saw the same tendency regarding ulcers in all trials where the number of ulcers where registered. In all trials with significant differences (trial 1, paper 2, trial 4 and 5 paper 3) the bacterial enforced groups had significantly fewer fish with ulcers compared to the control groups. Overall, this is a strong indication that bathing both Atlantic salmon and lumpfish with one or more of the three *Aliivibrio* spp. strains is positive for both Atlantic salmon and lumpfish regarding growth, feed utilization and number of ulcers and mortality.

For long time the mucosa of the gut has been seen as the most important point of contact between bacteria and the host. There are indications that bacteria

can pass through intact fish skin exemplified by the fish pathogens *Aliivibrio salmonicida* and *Moritella viscosa* using the skin as the portal of introduction to Atlantic salmon (Kashulin & Sørum, 2014; Løvoll et al. 2009; Karlsen et al. 2012). A common way of applying probiotic bacteria to fish has been to apply it together with the feed. Introducing probiotic bacteria into the rearing water is a relatively simple procedure with low costs. A user-friendly approach in introducing bacteria that will assist the fish in growing faster, utilizing feed better, developing fewer ulcers and having an improved survival rate is a key to continued use of probiotic bacteria in the aquaculture industry. The probiotic *A. balderis*, *A. njordis* and *A. nannie* used in the studies described in paper 2 and 3 are the three bacteria that the probiotic product manufactured by the company financing this PhD project, Previwo AS, are using in the commercial product Stembiont®. Stembiont® has been used in the water to more than 10 million of Atlantic salmon, lumpfish, rainbow trout, halibut and wrasses in commercial fish farming facilities. *A. balderis* and *A. nannie* were originally cultivated from kidney from Atlantic salmon and *A. njordis* and were originally cultivated from a mandible from an Atlantic salmon (Patent US, 2020). We have also cultivated two of the three *Aliivibrio* spp. from lumpfish as mentioned in paper 3.

The water-borne route can be the principal route for introducing bacteria to employ probiotic intervention strategies for farmed fish in the future. Adding probiotic bacteria to the rearing water simplifies the application mode for probiotic bacteria, compared to administration with the feed. There are for instance no concerns regarding loss of viability due to the low bactericidal pH in the stomach or deleterious effects of high processing temperature in feed manufacturing.

The process of attracting the microbiome in the early life stages is increasingly investigated (Ezenwa et al., 2012), and some scientists suppose that the process is mainly an active process. The process of getting the desired marine microbiome at transfer from freshwater to the sea being an active and not only a passive function also in Atlantic salmon may be linked to the observation that the scales of the Atlantic salmon smolts are loose and that the mucus cells decrease in numbers in connection with the smoltification process (O'Byrne-Ring et al., 2003). Many genes involving for example antigen presentation, production of cytokines and signal transducers and establishing cellular and humoral immunity of Atlantic

salmon are also down-regulated during the smoltification process, thereby allowing more bacterial exposure and transmission during this short period of transition from fresh to marine water (Johansson et al., 2016). In a vulnerable period of life, the salmon potentially become more admissible to bacterial invasion. Through long evolution it is unlikely that a down-regulation of the immune system, development of loose scales and fewer mucus cells would have been selected for if it had been negative to the salmon. It could be that the salmon makes itself more receptible to the new potential probiotic bacteria it meets in the saltwater. The growing knowledge of the importance of good bacteria in maintaining balance and homeostasis in eukaryotes supports the theory that fish, as Atlantic salmon, take risks, like turning down the immune system, to increase the probability to get suitable, acquired bacteria within the microbiome.

The fish both excretes and absorbs bacteria. A beneficial microbiome is therefore important not only for the fish individual itself, but also for other cohabiting fish of the same species or also individuals of other species of fish farmed together as with cleaner fish for sea lice control.

The cultivation of bacteria from lumpfish and Atlantic salmon was primarily done by using blood agar or Marine agar. Most of the bacteria, also in fish, are non-culturable on blood agar or Marine agar (Amann et al., 1995; Zhou et al., 2014). We have probably detected only some of the bacteria present in the lumpfish and Atlantic salmon from which we cultivated.

To identify the species of the bacteria we cultivated, we mostly used 16S *rRNA* gene analysis or Multi-locus Sequence Typing (MLST). The 16S *rRNA* gene consists of alternate conserved and highly variable regions, which increases its detection specificity. This also opens for the use of universal primers both forward and reversely located in the conserved areas. The 16S *rRNA* gene is part of the genetic background of the protein synthesis machinery and therefore present in all bacteria and becomes a universal target that has been employed for many years and with a resulting access to large number of bacterial 16S rDNA sequences in the databases. In addition, it evolves at relative constant rates, almost as a molecular clock, which allows determination of phylogenetic relationships. The cost of running sequencing analysis of the 16S *rRNA* gene is in addition relatively low. One problem with differentiation of bacteria based on the 16S *rRNA* gene sequence is that the16S

rRNA gene is highly conserved and it is sometimes difficult to distinguish between closely related bacterial species. For instance, the 16S *rRNA* gene in the probiotic *Aliivibrio* spp. we have used, is quite similar to other *Aliivibrio* spp. and the sequence will sometimes be mis-interpreted as belonging to another bacterium than it is isolated from, based on the 16S *rRNA* gene analysis. In general, the 16S *rRNA* gene is very conserved for the *Aliivibrio* spp. (Steinum et al., 2009). This will probably be the case for other closely related bacteria as well. In some differently taxonomically positioned bacterial species, the full length 16S *rRNA* genes are identical or close to identical. The discriminatory power of the 16S *rRNA* gene analysis is therefore limited and there are other technologies which are better suited in performing species classification of the bacteria analysed. House-keeping gene sequencing and MLSA tend to be more effective for distinguishing different bacteria and Multi-locus Sequence Typing (MLST), which compares internal sequences of several house-keeping genes is even more determinative. The most powerful tool for phylogenetic analysis is whole-genome sequence comparisons.

Lumpfish and Atlantic salmon are very different fish species. Bathing two such different species of fish as lumpfish and Atlantic salmon with the same probiotic bacteria gave almost the same results, better survival, protection against *Moritella viscosa* and increased growth, which is interesting. Some bacteria are likely to have a wide range of effects, both in terms of where they function and how they function. It has been indicated that vertebrates, including fish, have a complex resident bacterial microbiota that has co-evolved and developed delicate and intimate relationships benefiting both host and bacteria (Brugman et al., 2018; Kodio et al. 2020; Kostic et al., 2013; Maynard et al., 2012). The relationship of commensal bacteria and the host is long in a co-evolutionary sense. These relationships are important contributors to the development of regulatory systems of the host for instance to recognize friend or foe and to steer the physiological and immune systems. We are just beginning to understand the importance of this co-existence. The microbiota of fish is closely related to the surroundings (Egerton et al., 2018). It has been revealed that the vertebrate host often makes efforts to keep the right species of bacteria. The contact and colonization between fish and bacteria appears also to be an evolved and non-random process (Larsen et al., 2013; Schmidt et al., 2015). It is likely important for eukaryotes to get in contact with and be

colonized by the diverse and beneficial bacteria that promote good health. For fish living in close contact with the surrounding water this is not unlikely even more important than in terrestrial animals. Salmonid species, changing environment between so different habitats as freshwater and saltwater, have extraordinary challenges. When migrating from freshwater to seawater, the smolt encounter a very different bacterial environment and have an altered microbiome (Llewellyn et al., 2016). In aquaculture the contact with the bacteria and colonization of the microbiota can be problematic and unnatural. Aquaculture alters the living environment for the fish and its bacterial flora tremendously (Ramirez & Romero, 2017). Feeding, faeces, rearing temperature, RAS (Recirculating aquaculture systems) technology and water disinfection influence the bacteria in the rearing water making the bacteria in nature and in artificial rearing systems very different (Banerjee & Ray, 2017; de Carvalho, 2017; Lee et al., 2016; Moreno-Andres et al., 2018). Both the presence of feed and faeces in the rearing water result in an increase in dissolved organic matter (DOM) and disinfection of the intake water tends to result in selection of bacterial species that are opportunistic and have high growth rates. Furthermore, it produces systems with low bacterial diversity, low bacterial control and poor stability for perturbations (Vadstein et al., 2018). All these non-natural factors in aquaculture contribute to disturb the delicate symbiosis of fish and bacteria, and dysbiosis may occur (Brugman et al., 2018). As indicated in paper 1, 2 and 3 we believe it is very important for the fish to have an optimal microbiome. In modern aquaculture where the environment for the fish is very different from the fish`s natural environment, also regarding the bacterial species in the water, it can be challenging for the fish to acquire, develop and retain a good and beneficial microbiome. In nature, the microbiome has developed after thousands of years with coexistence of fish and bacteria in natural aquatic environments. The microbiome in wild fish and fish in aquaculture is reported to be significantly different (Ramirez & Romero, 2017). We think it is likely that the microbiome the fish acquires in aquaculture is not optimal. This potential suboptimal process of establishment of the microbiome starts early. Eggs kept in incubators in aquaculture are often heavily overgrown by a microflora that differ considerably from the natural environment in rivers and in the sea, and fish larvae are also ingesting bacteria by drinking water before active feeding is started (Olafsen, 2001). It is not unlikely that some of the

microbiome from the brood fish is transferred vertically via the egg, but much of the microbiome is acquired by the fish itself in the environment it habituates. As mentioned, fish that changes environment fundamentally during different life stages, as the anadromic Atlantic salmon does from freshwater to sea water, have special challenges regarding adaption of the microbiome. As described in paper 2, the transition period is even more important when we know that the immune system in the Atlantic salmon is turned down and the mucus layer is reduced in the vulnerable process of smoltification, when the smolt migrate to the new and potentially dangerous sea water environment. It is likely important that the microbiome bacteria the smolt meets in this vulnerable stage are mostly supportive of health and overall fitness. In modern aquaculture facilities this is not always the case, and we believe that the supply of natural probiotic bacteria as the *Aliivibrio* spp. described in paper 2 and 3 increases the possibility for the salmon to acquire a supportive bacterial community and microbiome. Adding *A. nannie*, *A. njordis* or *A. balderis* that are natural bacterial species collected from Atlantic salmon to the rearing water will likely contribute to improve the microbiome of the fish. The result will probably help the salmon to resist infectious diseases, grow better and be better suited to the environment at sea. A good opportunity to introduce the potential probiotic marine *Aliivibrio* spp. is probably prior to or during the period when the Atlantic salmon changes environment from fresh water to marine water. The physiological changes associated with smoltification may let the bacteria pass through the skin easier and the immune system may tolerate the potential probiotic aliivibrio bacteria since they are naturally occurring in seawater.

The addition of naturally occurring probiotic bacteria may stabilize and enhance the bacterial microbiota in the fish and diminish the level of dysbiosis and the potential of pathogenic bacteria to cause disease, making the fish healthier and improving food utilization. We think this is part of the explanation why addition of these bacteria revealed increased growth, improved feed conversion rate, less ulcer and made both the lumpfish and salmon more resistant to bacterial diseases as described in paper 2 and 3.

The behavioural observations from bathing both Atlantic salmon and lumpfish with the probiotic *Aliivibrio* spp. was that the fish schooled homogenously, became calm with no sign of stress, as mentioned in paper 2. We believe that both

the salmon and the lumpfish responded positively to the contact with the *Aliivibrio* spp. This supports the observations that it is a deterministic and active selection that drive fish microbiome assembly (Larsen et al., 2013; Schmidt et al., 2015).

In recent studies researchers have found that the microbiota of different species often resembles each other. Studies with germ-free zebrafish and germ-free mice given reciprocal transplantations of gut microbiota have revealed that microbial communities resemble their community of origin, but also that the relative abundance of the microbial species changes to resemble the normal gut microbial community of the recipient host. Investigations have also revealed that microbiota of herbivorous fish is closely related to the microbiota of mammals (Brugman et al., 2018). It is not surprising that many of the same bacterial pathogens make diseases in much more related species such as the teleosts Atlantic salmon and lumpfish, and also that we found the same potential probiotic bacteria in both lumpfish and Atlantic salmon. The probiotic *Aliivibrio* spp. we used in our trials demonstrated better protection against disease caused by *M. viscosa*, with less mortality and better growth in both Atlantic salmon and lumpfish. It will not surprise us if these probiotic *Aliivibrio* spp. also have positive effects regarding health and growth in other fish species with a marine life or a marine stage. As mentioned, implementation of the product Stembiont® with *A. njordis* and *A. balderis* has been applied with wrasse species and halibut also.

After treatment with florfenicol in trials 4 and 5 in paper 3, the performance regarding ulcer and mortality in the bacterial enhanced groups and the control groups became more similar. This was as expected. We know that the *Vibrio/Aliivibrio* spp. are sensitive to florfenicol (Felleskatalogen, 2020) and it is likely that the florfenicol given in trials 4 and 5 in paper 3 inactivated most of the probiotic *Aliivibrio* spp. present in the lumpfish affected by *M. viscosa*. Both the causative *M. viscosa* and the preventive *Aliivibrio* spp. were probably partly eliminated from the lumpfish due to the treatment with florfenicol, and therefore the positive effects of the *Aliivibrio* spp. partly disappeared as the *Aliivibrio* spp. were eradicated from the fish microbiota to some degree.

The mechanisms behind how the *Aliivibrio* spp. strains eventually help the fish to combat diseases, utilize food better and grow faster are unknown. We believe

that probiotic bacteria, such as the *Aliivibrio* spp. we have bathed or dipped the fish with as described in paper 2 and 3 could suppress or discourage other, pathogenic, bacteria. To not be defeated and have a positive effect on the fish, the probiotic bacteria should be recognized by the fish to be harmless (Lazado & Caipang, 2014). Why the probiotic *Aliivibrio* spp. probably do not activate the immune system and are recognized by the fish to be harmless is unknown. We know that the skin mucosa is an active immune-sensory surface in Atlantic salmon (Tadiso et al., 2011; Rombout et al., 2014). The *Aliivibrio* spp. may avoid the host immune system by muting of the gene expression as seen with *Al. salmonicida* (Bjelland et al., 2013) or it may be that the Toll-like receptors (TLR) of the fish ignore the microbe-associated molecular patterns (MAMP) of the probiotic *Aliivibrio* spp. as seen in some commensal bacterial species (Ausubel, 2005). The fish pathogen *Vibrio anguillarum* is using the lipopolysaccharides (LPS) of the outer membrane in avoiding phagocytosis by the skin epithelial cells in rainbow trout (Lindell et al. 2012). Mutant *V. anguillarum* with inactivated transport genes for LPS to the surface of the bacterial cell were not able to colonize the skin of rainbow trout. Not unlikely other systems are also involved. The reduced mortality in both lumpfish and Atlantic salmon attacked by *Moritella viscosa*, as described in paper 2 and 3, could be caused by the same inhibiting mechanism towards *M. viscosa* that *A. wodanis* performs. This is especially likely since the *A. nannie, A. balderis* and *A. njordis* are phylogenetically close to *A. wodanis*. We know that the presence of *A. wodanis* alters the gene expression of *M. viscosa*, perhaps by inhibiting bacteriocin activity (Hjerde et al., 2015; Karlsen et al 2014). We know that antibody responses in Atlantic salmon are shown to correlate with the antigen dose at least against some bacterial diseases (Romstad et al., 2013). The concentration of antibodies against *M. viscosa* from survivors in the trial affected by an outbreak of *M. viscosa* described in paper 2, was inversely proportional to the concentration of the probiotic bacteria in the bath, although the results were not significant ($p>0.05$). This is an indication that the probiotic protection against disease caused by *M. viscosa* was not based on an increased activity of the humoral immune defence but rather an indication that the probiotic *Aliivibrio* spp. to a certain extent defeated *M. viscosa* before the humoral immune system was triggered.

The microbial community of fish is closely linked to disease resistance by inhibiting, outcompeting and displacing potentially malevolent agents as bacteria (Brugman et al., 2018). In addition, some probiotic bacteria directly help the fish to become more robust and utilize the feed better by upregulation of genes responsible for physiological processes such as immune activity, pathogen protection and metabolism (Esser et al., 2018). It is well known that some of the bacterial quorum-sensing signals, the auto-inducers, are acting like hormones and have the ability to affect cells in their eukaryotic host (Bassler & Losick, 2006; Pacheco & Sperandio, 2009). As mentioned, most of the *Vibrio/Aliivibrio* spp. tested for the QS signals AHL and AI-2 produce both (Girard, 2019). It is not unlikely a combination of some of these actions that causes the beneficial effects of the probiotic *Aliivibrio* spp. used in the described trials in paper 2 and 3. More research remains to be done before we can explain the mechanisms of action of the potential probiotic *Aliivibrio* spp. So far, we have only investigated three of the potential probiotic bacteria that inhabit Atlantic salmon and lumpfish. There are probably many more.

Another discussion that should be considered with the growing knowledge of interacting bacteria is the disinfection of intake water for aquaculture sites. Disinfection of the intake water does not usually kill all the bacteria (de Carvalho, 2017; Moreno-Andres et al., 2018). In some cases, the pathogenic bacteria may be reduced but in other cases non-pathogenic beneficial bacteria may selectively be reduced and overall be too few to maintain a healthy balance compared to the pathogens both in the environment and inside the fish. UV-disinfection could further minimize the number of beneficial bacteria and negatively alter the bacterial flora of the fish or alter the balance between beneficial and pathogenic bacteria, making it easier for the pathogens to colonize and cause disease. In the site described in paper 1 and in trial 1 and 2 in paper 2, the fish were not exposed to seawater before being transferred to the described post-smolt sites. The land-based commercial aquaculture sites were also all flow-through facilities where the incoming water was UV-disinfected seawater pumped up from the fjord nearby. In addition to UV-disinfection, the water in one of the lumpfish facilities was also disinfected with ozone. Despite UV-disinfection of all the ingoing water with a UV-dose of more than 45mJ/cm^2, well above the national limit in Norway of 25 mJ/cm^2 (Veterinærinstituttet, 2017), some salmon were affected by potential pathogenic

bacteria as *T. dicentrarchi*. Also, some of the Atlantic salmon from trial 3 described in paper 2 and some of the lumpfish described in paper 3, despite UV treatment of incoming water, were also affected by potential pathogenic bacteria such as *M. viscosa, T. dicentrarchi, A. wodanis* and *V. splendidus*. It could be that the UV-disinfection or other methods of disinfecting the water in some cases make it easier for potential pathogenic bacteria to cause harm, because they have less competition and influence from beneficial bacteria. If we have knowledge of what bacteria that occur in the intake water, what interactions the bacteria have with each other and with the fish, it could in many cases possibly be a better option not to disinfect the water. Particularly when the bacterial population in the intake water mostly contains beneficial bacteria.

When bathing lumpfish and Atlantic salmon as a probiotic intervention with the *A. njordis*, *A. balderis* and *A. nannie* it was indicated that the seeding concentration in water should be more than 5 x 10^6 cfu per ml. Even though it seemed that it was a threshold before the bacteria added to the fish performed significant improvement to the fish in the trials described in paper 2 and 3, we also think that adding fewer bacteria to the water over a longer time will also be a positive intervention. As UV-disinfection or other disinfection procedures do not kill all the bacteria and could favour the relative survival of potential pathogenic bacteria, a continuous adding of probiotic bacteria to the rearing water after disinfection could make the microbiome of the fish healthier, preventing diseases and favour growth. This will probably be positive both in flow-through systems and Recirculating aquaculture systems (RAS)-systems.

Adding probiotic bacteria to the rearing water, such as the *Aliivibrio* spp. we used for bathing the lumpfish and the Atlantic salmon in our trials, may become one of the permanent preventive interventions in aquaculture of the future; as an aid in the prevention of diseases, improving animal welfare and for utilizing the feed better, thus making the overall aquaculture more sustainable and a help in taking nature back in aquaculture.

5. MAIN CONCLUSIONS

Tenacibaculum dicentrarchi causes acute disease with ulcers and mortality in Atlantic salmon in Norway.

Supply of non-pathogenic aliivibrio bacteria to the rearing water of Atlantic salmon, have probiotic effect making the fish more robust as evidenced by a reduction in mortality and ulcers, and an improved growth and contribute to feed utilization.

Supply of non-pathogenic aliivibrio bacteria to the rearing water of lumpfish, seem to have probiotic effect making the fish more robust as evidenced by a reduction in mortality and ulcers.

Supply of non-pathogenic aliivibrio bacteria to the rearing water of Atlantic salmon and lumpfish reduce mortality in outbreaks of disease caused by *Moritella viscosa*.

Concentration of non-pathogenic aliivibrio bacteria introduced into the rearing water in single bath treatment matters regarding manifestation of the probiotic effect, and it is indicated that the concentration of probiotic aliivibrio bacteria in the rearing water during bathing should be at least $5 \text{ X } 10^6$ cfu per ml.

6. FUTURE PERSPECTIVES

Although we see that *Aliivibrio* spp. (*A. njordis* NCIMB 42593 (VL1), *A. balderis* NCIMB 42592 (VL2) and *A. nannie* NCIMB 42594 (VL3)) have probiotic effects in Atlantic salmon, lumpfish and probably in other species, we have little knowledge about the mechanisms of action. The mechanisms behind the interactions between bacteria and bacteria and eukaryote are largely unknown.

Still very many Atlantic salmon, lumpfish and other fish continue dying in cages both in Norway and elsewhere in the world, not least because of ulcers and chronic diseases. There are probably more bacteria causing disease than those which are currently recognized as disease-causing. Considerably more research is needed in this field of knowledge with increased theoretical understanding of the interactions between the different bacteria including those that are probiotic. A lot of research to find out about this remains to be done especially since the utility and safety of antibiotic compounds is limited. New advances in bacterial disease prophylaxis using preventative approaches and foundational research on bacterial microbiology and the host as a holobiont is a key.

New knowledge about bacteria that result in disease, and about interactions between the bacteria and between the bacteria and the host will probably also make us able to make far more effective interactive tools as probiotics and vaccines, particularly against chronic diseases caused by bacteria.

7. REFERENCES

Abrudan, M. I., Smakman, F., Grimbergen, A. J., Westhoff, S., Miller, E. L., van Wezel, G. P., & Rozen, D. E. (2015). Socially mediated induction and suppression of antibiosis during bacterial coexistence. *Proc Natl Acad Sci U S A, 112*(35), 11054-11059. doi:10.1073/pnas.1504076112

Adams, A. (2019). Progress, Challenges And Opportunities In Fish Vaccine Development. *Fish Shellfish Immunol*. doi:10.1016/j.fsi.2019.04.066

Ahmed, N., Thompson, S., & Glaser, M. (2019). Global Aquaculture Productivity, Environmental Sustainability, and Climate Change Adaptability. *Environ Manage, 63*(2), 159-172. doi:10.1007/s00267-018-1117-3

Ahmer, B. M., van Reeuwijk, J., Timmers, C. D., Valentine, P. J., & Heffron, F. (1998). Salmonella typhimurium encodes an SdiA homolog, a putative quorum sensor of the LuxR family, that regulates genes on the virulence plasmid. *J Bacteriol, 180*(5), 1185-1193.

Akhter, N., Wu, B., Memon, A. M., & Mohsin, M. (2015). Probiotics and prebiotics associated with aquaculture: A review. *Fish Shellfish Immunol, 45*(2), 733-741. doi:10.1016/j.fsi.2015.05.038

Amann, R. I., Ludwig, W., & Schleifer, K. H. (1995). Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol Rev, 59*(1), 143-169.

Aryana, K. J., & Olson, D. W. (2017). A 100-Year Review: Yogurt and other cultured dairy products. *J Dairy Sci, 100*(12), 9987-10013. doi:10.3168/jds.2017-12981

Ast, J. C., Urbanczyk, H., & Dunlap, P. V. (2009). Multi-gene analysis reveals previously unrecognized phylogenetic diversity in *Aliivibrio*. *Syst Appl Microbiol, 32*(6), 379-386. doi:10.1016/j.syapm.2009.04.005

Aunsmo, A., Bruheim, T., Sandberg, M., Skjerve, E., Romstad, S., Larssen, R. B. (2008). Methods for investigating patterns of mortality and quantifying cause-specific mortality in sea farmed Atlantic salmon, *Salmo salar*. Diseases of Aquatic Organisms 81: 99-107.

Austin, B., Baudet, E., & M. S, S. (2006). Inhibition of bacteria fish pathogens by *Tetraselmis suecica*. *J Fish Dis, 15*, 55-61. doi:10.1111/j.1365-2761.1992.tb00636.x

Ausubel, F. M. (2005). Are innate immune signaling pathways in plants and animals conserved? *Nat Immunol, 6*(10), 973-979. doi:10.1038/ni1253

Avendano-Herrera, R., Irgang, R., Sandoval, C., Moreno-Lira, P., Houel, A., Duchaud, E., Ilardi, P. (2016). Isolation, Characterization and Virulence Potential of T*enacibaculum dicentrarchi* in Salmonid Cultures in Chile. *Transbound Emerg Dis, 63*(2), 121-126. doi:10.1111/tbed.12464

Avendano-Herrera, R., Irgang, R., & Tapia-Cammas, D. (2018). PCR procedure for detecting the fish pathogen T*enacibaculum dicentrarchi*. *J Fish Dis, 41*(4), 715-719. doi:10.1111/jfd.12767

Avendano-Herrera, R., Toranzo, A. E., & Magarinos, B. (2006). Tenacibaculosis infection in marine fish caused by *Tenacibaculum maritimum*: a review. *Dis Aquat Organ, 71*(3), 255-266. doi:10.3354/dao071255

Backhed, F. (2019). Shining light on microbial signaling to distant organs. *Proc Natl Acad Sci U S A, 116*(16), 7617-7619. doi:10.1073/pnas.1902664116

Bakke-McKellep, A. M., Penn, M. H., Salas, P. M., Refstie, S., Sperstad, S., Landsverk, T., Krogdahl, A. (2007). Effects of dietary soyabean meal, inulin and oxytetracycline on intestinal microbiota and epithelial cell stress, apoptosis and proliferation in the teleost Atlantic salmon (*Salmo salar L.*). *Br J Nutr, 97*(4), 699-713. doi:10.1017/s0007114507381397

Balcazar, J. L., de Blas, I., Ruiz-Zarzuela, I., Cunningham, D., Vendrell, D., & Muzquiz, J. L. (2006). The role of probiotics in aquaculture. *Vet Microbiol, 114*(3-4), 173-186. doi:10.1016/j.vetmic.2006.01.009

Balzan S, de Almeida Quadros C, de Cleva R, Zilberstein B, Cecconello I. Bacterial translocation: overview of mechanisms and clinical impact. *J Gastroenterol Hepatol*. 2007 Apr;22(4):464-71. doi: 10.1111/j.1440-1746.2007.04933.x. PMID: 17376034.

Banerjee, G., & Ray, A. K. (2017). The advancement of probiotics research and its application in fish farming industries. *Res Vet Sci, 115*, 66-77. doi:10.1016/j.rvsc.2017.01.016

Bang, C., Dagan, T., Deines, P., Dubilier, N., Duschl, W. J., Fraune, S., . Bosch, T. C. G. (2018). Metaorganisms in extreme environments: do microbes play a role in organismal adaptation? *Zoology (Jena), 127*, 1-19. doi:10.1016/j.zool.2018.02.004

Bassler, B. L., & Losick, R. (2006). Bacterially speaking. *Cell, 125*(2), 237-246. doi:10.1016/j.cell.2006.04.001

Baumler, A. J., & Sperandio, V. (2016). Interactions between the microbiota and pathogenic bacteria in the gut. *Nature, 535*(7610), 85-93. doi:10.1038/nature18849

Baxa, D. V., Kawai, K., & Kusuda, R. (1987). Experimental-infection of flexibacter-maritimus in black-sea bream (*Acanthopagrus-schlegeli*) FRY. *Fish Pathology, 22*(2), 105-109. doi:10.3147/jsfp.22.105

Baxby, D. (1999). Edward Jenner's Inquiry; a bicentenary analysis. *Vaccine, 17*(4), 301-307.

Beeston, A. L., & Surette, M. G. (2002). pfs-dependent regulation of autoinducer 2 production in *Salmonella enterica* serovar Typhimurium. *J Bacteriol, 184*(13), 3450-3456. doi:10.1128/jb.184.13.3450-3456.2002

Belkaid, Y., & Hand, T. W. (2014). Role of the microbiota in immunity and inflammation. *Cell, 157*(1), 121-141. doi:10.1016/j.cell.2014.03.011

Benediktsdottir, E., Verdonck, L., Sproer, C., Helgason, S., & Swings, J. (2000). Characterization of *Vibrio viscosus* and *Vibrio wodanis* isolated at different geographical locations: a proposal for reclassification of *Vibrio viscosus* as *Moritella viscosa* comb. nov. *Int J Syst Evol Microbiol, 50 Pt 2*, 479-488. doi:10.1099/00207713-50-2-479

Berg RD, Garlington AW. Translocation of certain indigenous bacteria from the gastrointestinal tract to the mesenteric lymph nodes and other organs in a gnotobiotic mouse model. Infect Immun. 1979 Feb;23(2):403-11. doi: 10.1128/IAI.23.2.403-411.1979. PMID: 154474; PMCID: PMC414179.

Bhandal, J., Hayashi, K., Kim, S.-Y., Klein, M., Wong, A., Toupadakis, C.A., Muir, P. and Yellowley, C.E. (2013), Detection of bacterial DNA by PCR in dogs with stifle pathology. Veterinary Surgery, 42: 814-818. doi:10.1111/j.1532-950X.2013.12038.x

Bjelland, A. M., Fauske, A. K., Nguyen, A., Orlien, I. E., Ostgaard, I. M., & Sørum, H. (2013). Expression of *Vibrio salmonicida* virulence genes and immune response parameters in experimentally challenged Atlantic salmon (*Salmo salar L.*). *Front Microbiol, 4*, 401. doi:10.3389/fmicb.2013.00401

Boutin, S., Bernatchez, L., Audet, C., & Derome, N. (2012). Antagonistic effect of indigenous skin bacteria of brook charr (*Salvelinus fontinalis*) against *Flavobacterium columnare* and F. psychrophilum. *Vet Microbiol, 155*(2-4), 355-361. doi:10.1016/j.vetmic.2011.09.002

Boyer, M., & Wisniewski-Dye, F. (2009). Cell-cell signalling in bacteria: not simply a matter of quorum. *FEMS Microbiol Ecol, 70*(1), 1-19. doi:10.1111/j.1574-6941.2009.00745.x

Bravo, J. A., Forsythe, P., Chew, M. V., Escaravage, E., Savignac, H. M., Dinan, T. G.,Cryan, J. F. (2011). Ingestion of Lactobacillus strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. *Proc Natl Acad Sci U S A, 108*(38), 16050-16055. doi:10.1073/pnas.1102999108

Brocklebank, J. R. (1998). Ulcerative dermatitis caused by *Aeromonas salmonicida spp. salmonicida* in farmed Atlantic salmon in British Columbia. *The Canadian Veterinary Journal, 39*(2), 110.

Brugman, S., Ikeda-Ohtsubo, W., Braber, S., Folkerts, G., Pieterse, C. M. J., & Bakker, P. (2018). A Comparative Review on Microbiota Manipulation: Lessons From Fish, Plants, Livestock, and Human Research. *Front Nutr, 5*, 80. doi:10.3389/fnut.2018.00080

Burtseva, O., Kublanovskaya, A., Fedorenko, T., Lobakova, E., Chekanov, K. (2021). Gut microbiome of the White Sea fish revealed by 16S rRNA metabarcoding, Aquaculture,Volume 533, 2021, 736175, ISSN 0044-8486,https://doi.org/10.1016/j.aquaculture.2020.736175.

Butt, R. L., & Volkoff, H. (2019). Gut Microbiota and Energy Homeostasis in Fish. *Front Endocrinol (Lausanne), 10*, 9. doi:10.3389/fendo.2019.00009

Byrd, A. L., & Segre, J. A. (2016). Infectious disease. Adapting Koch's postulates. *Science, 351*(6270), 224-226. doi:10.1126/science.aad6753

Cahill, M. M. (1990). Bacterial flora of fishes: A review. *Microb Ecol, 19*(1), 21-41. doi:10.1007/bf02015051

Camilli, A., & Bassler, B. L. (2006). Bacterial small-molecule signaling pathways. *Science, 311*(5764), 1113-1116. doi:10.1126/science.1121357

Carbajal-Gonzalez, M. T., Fregeneda-Grandes, J. M., Suarez-Ramos, S., Rodriguez Cadenas, F., & Aller-Gancedo, J. M. (2011). Bacterial skin flora variation and in vitro inhibitory activity against *Saprolegnia parasitica* in brown and rainbow trout. *Dis Aquat Organ, 96*(2), 125-135. doi:10.3354/dao02391

Cavarretta I, Ferrarese R, Cazzaniga W, et al. (2017). The Microbiome of the Prostate Tumor Microenvironment. Eur Urol. 2017;72(4):625-631. doi:10.1016/j.eururo.2017.03.029

Chakraborty, S., Cao, T., Hossain, A., Gnanagobal, H., Vasquez, I., Boyce, D., & Santander, J. (2019). Vibrogen-2 vaccine trial in lumpfish (*Cyclopterus lumpus*) against *Vibrio anguillarum*. *J Fish Dis, 42*(7), 1057-1064. doi:10.1111/jfd.13010

Chen, X., Schauder, S., Potier, N., Van Dorsselaer, A., Pelczer, I., Bassler, B. L., & Hughson, F. M. (2002). Structural identification of a bacterial quorum-sensing signal containing boron. *Nature, 415*(6871), 545-549. doi:10.1038/415545a

Chiarello, M., Villeger, S., Bouvier, C., Bettarel, Y., & Bouvier, T. (2015). High diversity of skin-associated bacterial communities of marine fishes is promoted by their high variability among body parts, individuals and species. *FEMS Microbiol Ecol, 91*(7). doi:10.1093/femsec/fiv061

Citrome, L. (2018). Happy Birthday Ignac Semmelweiss! Now, Let's All Wash Our Hands! *Int J Clin Pract, 72*(10), e13256. doi:10.1111/ijcp.13256

Connell, J. L., Kim, J., Shear, J. B., Bard, A. J., & Whiteley, M. (2014). Real-time monitoring of quorum sensing in 3D-printed bacterial aggregates using scanning electrochemical microscopy. *Proc Natl Acad Sci U S A, 111*(51), 18255-18260. doi:10.1073/pnas.1421211111

Crabbe, A., Jensen, P. O., Bjarnsholt, T., & Coenye, T. (2019). Antimicrobial Tolerance and Metabolic Adaptations in Microbial Biofilms. *Trends Microbiol*. doi:10.1016/j.tim.2019.05.003

Crumlish, M., Thanh, P. C., Koesling, J., Tung, V. T., & Gravningen, K. (2010). Experimental challenge studies in Vietnamese catfish, *Pangasianodon hypophthalmus* (Sauvage), exposed to E*dwardsiella ictalu*ri and *Aeromonas hydrophila*. *J Fish Dis, 33*(9), 717-722. doi:10.1111/j.1365-2761.2010.01173.x

Cummins J, Tangney M. Bacteria and tumours: causative agents or opportunistic inhabitants? *Infect Agent Cancer.* 2013 Mar 28;8(1):11. doi: 10.1186/1750-9378-8-11. PMID: 23537317; PMCID: PMC3668256.

de Carvalho, C. (2017). Biofilms: Microbial Strategies for Surviving UV Exposure. *Adv Exp Med Biol, 996*, 233-239. doi:10.1007/978-3-319-56017-5_19

Decho, A. W., Frey, R. L., & Ferry, J. L. (2011). Chemical challenges to bacterial AHL signaling in the environment. *Chem Rev, 111*(1), 86-99. doi:10.1021/cr100311q

Diard, M., & Hardt, W. D. (2017). Evolution of bacterial virulence. *FEMS Microbiol Rev, 41*(5), 679-697. doi:10.1093/femsre/fux023

Dimitroglou, A., Merrifield, D. L., Carnevali, O., Picchietti, S., Avella, M., Daniels, C., Davies, S. J. (2011). Microbial manipulations to improve fish health and production--a Mediterranean perspective. *Fish Shellfish Immunol, 30*(1), 1-16. doi:10.1016/j.fsi.2010.08.009

Dominguez-Bello, M. G., Godoy-Vitorino, F., Knight, R., & Blaser, M. J. (2019). Role of the microbiome in human development. *Gut, 68*(6), 1108-1114. doi:10.1136/gutjnl-2018-317503

Dong, H., Nguyen, V., Phiwsaiya, K., Gangnonngiw, W., Withyachumnarnkul, B., Rodkhum, C., & Senapin, S. (2015). Concurrent infections of *Flavobacterium columnare* and Edwardsiella ictaluri in striped catfish, *Pangasianodon hypophthalmus* in Thailand (Vol. 448).

Dunlap, P. V., & Greenberg, E. P. (1985). Control of *Vibrio fischeri* luminescence gene expression in *Escherichia coli* by cyclic AMP and cyclic AMP receptor protein. *J Bacteriol, 164*(1), 45-50.

Dunlap, P. V., & Greenberg, E. P. (1988). Control of *Vibrio fischeri* lux gene transcription by a cyclic AMP receptor protein-luxR protein regulatory circuit. *J Bacteriol, 170*(9), 4040-4046. doi:10.1128/jb.170.9.4040-4046.1988

Eberhard, A., Burlingame, A. L., Eberhard, C., Kenyon, G. L., Nealson, K. H., & Oppenheimer, N. J. (1981). Structural identification of autoinducer of *Photobacterium fischeri* luciferase. *Biochemistry, 20*(9), 2444-2449. doi:10.1021/bi00512a013

Egan S and Gardiner M (2016) Microbial Dysbiosis: Rethinking Disease in Marine Ecosystems. Front. Microbiol. 7:991. doi: 10.3389/fmicb.2016.00991

Egerton, S., Culloty, S., Whooley, J., Stanton, C., & Ross, R. P. (2018). The Gut Microbiota of Marine Fish. *Front Microbiol, 9*, 873. doi:10.3389/fmicb.2018.00873

Egidius, E., Wiik, R., Andersen, K., Hoff, K. A., & Hjeltnes, B. (1986). *Vibrio salmonicida sp.* nov., a New Fish Pathogen. *36*(4), 518-520. doi:doi:10.1099/00207713-36-4-518

Einarsdottir, T., Sigurdardottir, H., Bjornsdottir, T. S., & Einarsdottir, E. (2018). *Moritella viscosa* in lumpfish (*Cyclopterus lumpus*) and Atlantic salmon (*Salmo salar*). *J Fish Dis*. doi:10.1111/jfd.12884

El-Kholy, A., El-Shinawy, A. Meshref, A. K, Screening of antagonistic activity of probiotic bacteria against some food-borne pathogensJ. Appl. Environ. Microbiol., 2 (2) (2014), pp. 53-60

Ellul, R. M., Walde, C., Haugland, G. T., Wergeland, H., & Ronneseth, A. (2018). Pathogenicity of *Pasteurella* sp. in lumpsuckers (*Cyclopterus lumpus L.*). *J Fish Dis*. doi:10.1111/jfd.12905

Engebrecht, J., Nealson, K., & Silverman, M. (1983). Bacterial bioluminescence: isolation and genetic analysis of functions from *Vibrio fischeri*. *Cell, 32*(3), 773-781.

Engebrecht, J., & Silverman, M. (1984). Identification of genes and gene products necessary for bacterial bioluminescence. *Proc Natl Acad Sci U S A, 81*(13), 4154-4158. doi:10.1073/pnas.81.13.4154

Esser, D., Lange, J., Marinos, G., Sieber, M., Best, L., Prasse, D., Sommer, F. (2018). Functions of the Microbiota for the Physiology of Animal Metaorganisms. *J Innate Immun*, 1-12. doi:10.1159/000495115

Estensoro, I., Jung-Schroers, V., Alvarez-Pellitero, P., Steinhagen, D., & Sitja-Bobadilla, A. (2013). Effects of *Enteromyxum leei* (*Myxozoa*) infection on gilthead sea bream (*Sparus aurata*) (Teleostei) intestinal mucus: glycoprotein profile and bacterial adhesion. *Parasitol Res, 112*(2), 567-576. doi:10.1007/s00436-012-3168-3

Ezenwa, V. O., Gerardo, N. M., Inouye, D. W., Medina, M., & Xavier, J. B. (2012). Microbiology. Animal behavior and the microbiome. *Science, 338*(6104), 198-199. doi:10.1126/science.1227412

Falcinelli, S., Picchietti, S., Rodiles, A., Cossignani, L., Merrifield, D. L., Taddei, A. R., Carnevali, O. (2015). *Lactobacillus rhamnosus* lowers zebrafish lipid content by changing gut microbiota and host transcription of genes involved in lipid metabolism. *Sci Rep, 5*, 9336. doi:10.1038/srep09336

FAO 1. (2018). FAO yearbook. Fishery and Aquaculture Statistics 2016/FAO annuaire. Statistiques des pêches et de l'aquaculture 2016/FAO anuario. Estadísticas de pesca y acuicultura 2016. Rome/Roma. 104pp.

FAO 2. (2018). The State of World Fisheries and Aquaculture 2018 - Meeting the sustainable development goals. Rome. Licence: CC BY-NC-SA 3.0 IGO. http://www.fao.org/state-of-fisheries-aquaculture).

Feckaninová, A., Koscková. J., Mudronová, D., Popelka, P., & Toropilová, J. (2017). The use of probiotic bacteria against *Aeromonas* infections in salmonid aquaculture. *Aquaculture*, 469, 1-8.

Felleskatalogen (2020). https://www.felleskatalogen.no/medisin-vet/floraqpharma-vet-skretting-559156

Fernandez-Alvarez, C., & Santos, Y. (2018). Identification and typing of fish pathogenic species of the genus *Tenacibaculum. Appl Microbiol Biotechnol, 102*(23), 9973-9989. doi:10.1007/s00253-018-9370-1

Foey A., Picchietti S.(2014). Immune defenses of teleost fish D. Merrifield, E. Ringø (Eds.), Aquaculture Nutrition: Gut Health, Probiotics and Prebiotics, John Wiley & Sons, Ltd., Chichester, UK (2014), 10.1002/9781118897263.ch2

Frans, I., Michiels, C. W., Bossier, P., Willems, K. A., Lievens, B., & Rediers, H. (2011). *Vibrio anguillarum* as a fish pathogen: virulence factors, diagnosis and prevention. *J Fish Dis, 34*(9), 643-661. doi:10.1111/j.1365-2761.2011.01279.x

Fujimoto, M., Lovett, B., Angoshtari, R., Nirenberg, P., Loch, T. P., Scribner, K. T., & Marsh, T. L. (2018). Antagonistic Interactions and Biofilm Forming Capabilities Among Bacterial Strains Isolated from the Egg Surfaces of Lake Sturgeon (*Acipenser fulvescens*). *Microb Ecol, 75*(1), 22-37. doi:10.1007/s00248-017-1013-z

Gajardo, K., Rodiles, A., Kortner, T. M., Krogdahl, A., Bakke, A. M., Merrifield, D. L., & Sørum, H. (2016). A high-resolution map of the gut microbiota in Atlantic salmon (*Salmo salar*): A basis for comparative gut microbial research. *Sci Rep, 6*, 30893. doi:10.1038/srep30893

Gaudier E, Hoebler C. (2006). Rôles physiologiques des mucines dans la barrière colique [Physiological role of mucins in the colonic barrier integrity]. Gastroenterol Clin Biol. 2006;30(8-9):965-974. doi:10.1016/s0399-8320(06)73358-9

Girard, L. (2019). Quorum sensing in Vibrio spp.: the complexity of multiple signalling molecules in marine and aquatic environments. *Crit Rev Microbiol*, 1-21. doi:10.1080/1040841x.2019.1624499

Gram, L., Melchiorsen, J., Spanggaard, B., Huber, I., & Nielsen, T. F. (1999). Inhibition of *Vibrio anguillarum* by P*seudomonas fluorescens* AH2, a possible probiotic treatment of fish. *Appl Environ Microbiol, 65*(3), 969-973.

Gribanov, E. D., & Potapchuk, T. B. (1996). [The classic work of Girolamo Fracastoro (on the 450th anniversary of the publication of his book "De contagione et de contagiosis morbis et curatione")]. *Voen Med Zh, 317*(8), 74-76.

Grove, S., Wiik-Nielsen, C. R., Lunder, T., Tunsjø, H. S., Tandstad, N. M., Reitan, L. J., Colquhoun, D. J. (2010). Previously unrecognised division within *Moritella viscosa* isolated from fish farmed in the North Atlantic. *Dis Aquat Organ, 93*(1), 51-61. doi:10.3354/dao02271

Gudding, R., & Van Muiswinkel, W. B. (2013). A history of fish vaccination: science-based disease prevention in aquaculture. *Fish Shellfish Immunol, 35*(6), 1683-1688. doi:10.1016/j.fsi.2013.09.031

Guerrero, R., Margulis, L., & Berlanga, M. (2013). Symbiogenesis: the holobiont as a unit of evolution. *Int Microbiol, 16*(3), 133-143. doi:10.2436/20.1501.01.188

Haas, L. F. (1992). Antoni van Leeuwenhoek 1632-1723. *J Neurol Neurosurg Psychiatry, 55*(4), 251. doi:10.1136/jnnp.55.4.251

Habib, C., Houel, A., Lunazzi, A., Bernardet, J. F., Olsen, A. B., Nilsen, H., . Duchaud, E. (2014). Multilocus sequence analysis of the marine bacterial genus *Tenacibaculum* suggests parallel evolution of fish pathogenicity and endemic colonization of aquaculture systems. *Appl Environ Microbiol, 80*(17), 5503-5514. doi:10.1128/aem.01177-14

Hai, N. V. (2015a). Research findings from the use of probiotics in tilapia aquaculture: A review. *Fish Shellfish Immunol, 45*(2), 592-597. doi:10.1016/j.fsi.2015.05.026

Hai, N. V. (2015b). The use of probiotics in aquaculture. *J Appl Microbiol, 119*(4), 917-935. doi:10.1111/jam.12886

Hammer, T. J., Sanders, J. G., & Fierer, N. (2019). Not all animals need a microbiome. *FEMS Microbiol Lett, 366*(10). doi:10.1093/femsle/fnz117

Hao, Y., Winans, S. C., Glick, B. R., & Charles, T. C. (2010). Identification and characterization of new LuxR/LuxI-type quorum sensing systems from metagenomic libraries. *Environ Microbiol, 12*(1), 105-117. doi:10.1111/j.1462-2920.2009.02049.x

Has, L. F. (1991). Avicenna or Ibn Sina (980-1036 AD). *J Neurol Neurosurg Psychiatry, 54*(8), 682. doi:10.1136/jnnp.54.8.682

Health Canada. Accepted Claims about the Nature of Probiotic Microorganisms in Food. Health Canada [online], http://www.hc-sc.gc.ca/fn-an/label-etiquet/claims-reclam/probiotics_claims-allegations_probiotiques-eng.php%20(2009).

Hill, C. et al. Nat. Rev. Gastroenterol. Hepatol. 11, 506–514 (2014); published online 10 June 2014; doi:10.1038/nrgastro.2014.66

Hjeltnes.B, Bang Jensen B, Bornø G, Haukaas A, Walde C S (red), Fiskehelserapporten 2018

Hjerde, E., Karlsen, C., Sørum, H., Parkhill, J., Willassen, N. P., & Thomson, N. R. (2015). Co-cultivation and transcriptome sequencing of two co-existing fish pathogens *Moritella viscosa* and *Aliivibrio wodanis*. *BMC Genomics, 16*, 447. doi:10.1186/s12864-015-1669-z

Holben, W. E., Williams, P., Gilbert, M., Saarinen, M., Sarkilahti, L. K., & Apajalahti, J. H. (2002). Phylogenetic analysis of intestinal microflora indicates a novel *Mycoplasma* phylotype in farmed and wild salmon. *Microb Ecol, 44*(2), 175-185. doi:10.1007/s00248-002-1011-6

Holm, K., & Jørgensen, T. (2006). A successful vaccination of Atlantic salmon, *Salmo salar L.*, against 'Hitra disease' or coldwater vibriosis. *J Fish Dis, 10*, 85-90. doi:10.1111/j.1365-2761.1987.tb00723.x

Hooper, L. V., Littman, D. R., & Macpherson, A. J. (2012). Interactions between the microbiota and the immune system. *Science, 336*(6086), 1268-1273. doi:10.1126/science.1223490

Hoseinifar, S. H., Sun, Y. Z., Wang, A., & Zhou, Z. (2018). Probiotics as Means of Diseases Control in Aquaculture, a Review of Current Knowledge and Future Perspectives. *Front Microbiol, 9*, 2429. doi:10.3389/fmicb.2018.02429

Hovda, M., Fontanillas, R., McGurk, C., Obach, & Rosnes, J. T. (2012). *Seasonal variations in the intestinal microbiota of farmed Atlantic salmon (Salmo salar L.)* (Vol. 43).

Hughes, D. T., & Sperandio, V. (2008). Inter-kingdom signalling: communication between bacteria and their hosts. *Nat Rev Microbiol, 6*(2), 111-120. doi:10.1038/nrmicro1836

Human Microbiome Project Consortium (2012). A framework for human microbiome research. Nature 486(7402). 207-221.

Hunter, M. C., Smith, R. G., Schipanski, M. E., Atwood, L. W., & Mortensen, D. (2017). Agriculture in 2050: Recalibrating targets for sustainable intensification. BioScience, 67(4), 386-391. https://doi.org/10.1093/biosci/bix010

Iida N, Dzutsev A, Stewart CA, Smith L, Bouladoux N, Weingarten RA, Molina DA, Salcedo R, Back T, Cramer S, Dai RM, Kiu H, Cardone M, Naik S, Patri AK, Wang E, Marincola FM, Frank KM, Belkaid Y, Trinchieri G, Goldszmid RS. Commensal bacteria control cancer response to therapy by modulating the tumor microenvironment. Science. 2013 Nov 22;342 (6161):967-70. doi: 10.1126/science.1240527. PMID: 24264989; PMCID: PMC6709532.

Irianto, A., & Austin, B. (2003). Use of dead probiotic cells to control furunculosis in rainbow trout, *Oncorhynchus mykiss* (Walbaum). *J Fish Dis, 26*(1), 59-62.

Johansson, L. H., Timmerhaus, G., Afanasyev, S., Jorgensen, S. M., & Krasnov, A. (2016). Smoltification and seawater transfer of Atlantic salmon (*Salmo salar L.*) is associated with systemic repression of the immune transcriptome. *Fish Shellfish Immunol, 58*, 33-41. doi:10.1016/j.fsi.2016.09.026

Jones SRM, Long A, MacWilliams C, Polinski M, Garver K. Factors associated with severity of naturally occurring piscirickettsiosis in netpen- and tank-reared juvenile Atlantic salmon at a research aquarium in western Canada. J Fish Dis. 2020;43(1):49-55. doi:10.1111/jfd.13102)/.

Kapp JP. Microorganisms as antineoplastic agents in CNS tumors. Arch Neurol. 1983 Oct;40(10):637-42. doi: 10.1001/archneur.1983.04050090073012. PMID: 6688521.

Karlsen, C., Ottem, K. F., Brevik, O. J., Davey, M., Sørum, H., & Winther-Larsen, H. C. (2017). The environmental and host-associated bacterial microbiota of Arctic seawater-farmed Atlantic salmon with ulcerative disorders. *J Fish Dis, 40*(11), 1645-1663. doi:10.1111/jfd.12632

Karlsen, C., Sørum, H., Willassen N. P., & Åsbakk, K. (2012). *Moritella viscosa* bypasses Atlantic salmon epidermal keratocyte clearing activity and might use skin surfaces as a port of infection. Vet Microbiol. 2012 Jan 27;154(3-4):353-62. doi: 10.1016/j.vetmic.2011.07.024. Epub 2011 Jul 30. PMID: 21862244

Karlsen, C., Vanberg, C., Mikkelsen, H., & Sørum, H. (2014). Co-infection of Atlantic salmon (*Salmo salar*), by *Moritella viscosa* and *Aliivibrio wodanis,* development of disease and host colonization. *Vet Microbiol, 171*(1-2), 112-121. doi:10.1016/j.vetmic.2014.03.011

Kashulin, A., & Sørum, H. (2014). A novel in vivo model for rapid evaluation of *Aliivibrio salmonicida* infectivity in Atlantic salmon. *Aquaculture, 420*, 112-118. doi:10.1016/j.aquaculture.2013.10.025

Kelly, C., & Salinas, I. (2017). Under Pressure: Interactions between Commensal Microbiota and the Teleost Immune System. *Front Immunol, 8*, 559. doi:10.3389/fimmu.2017.00559

Kodio, A., Menu, E., & Ranque, S. (2020). Eukaryotic and prokaryotic microbiota interactions. *Microorganisms*, 8, 2018; doi:10.3390/microorganisms8122018

Korkea-aho TL, Partanen JM, Kukkonen JV, Taskinen J. Hypoxia increases intensity of epidermal papillomatosis in roach *Rutilus rutilus*. Dis Aquat Organ. 2008;78(3):235-241. doi:10.3354/dao01876

Kostic, A. D., Howitt, M. R., & Garrett, W. S. (2013). Exploring host-microbiota interactions in animal models and humans. *Genes Dev, 27*(7), 701-718. doi:10.1101/gad.212522.112

Kotob, M. H., Menanteau-Ledouble, S., Kumar, G., Abdelzaher, M., & El-Matbouli, M. (2016). The impact of co-infections on fish: a review. *Vet Res, 47*(1), 98. doi:10.1186/s13567-016-0383-4

Kozasa, M. (1986). Toyocerin *(Bacillus toyoi)* as growth promotor for animal feeding. *Microbiol Alim Nutr* (Vol.4), 121-124.

Koziel J. & Potempa J. (2013). Protease-armed bacteria in the skin. *Cell Tissue Res*. 2013;351(2):325-337. doi:10.1007/s00441-012-1355-2)

Krasnov A, Moghadam H, Larsson T, Afanasyev S, Mørkøre T. (2016). Gene expression profiling in melanised sites of Atlantic salmon fillets. Fish Shellfish Immunol. 2016;55:56-63. doi:10.1016/j.fsi.2016.05.012.

Krogdahl Å., Hemre G.I., Mommsen T.P. (2005) Carbohydrates in fish nutrition: digestion and absorption in postlarval stages. Aquacult. Nutr., 11 (2005), pp. 103-122

Kuebutornye, F. K. A., Abarike, E. D., Lu, Y., Hlordzi, V., Sakyi, M. E., Afriyie, G., Wang, Z. Li, Y., & Xie, C. X. (2020) Mechanisms and the role of probiotic *Bacillus* in mitigating fish pathogens in aquaculture. *Fish Physiol Biochem*, 46: 819-841.

Kumar, G., Menanteau-Ledouble, S., Saleh, M., & El-Matbouli, M. (2015). *Yersinia ruckeri,* the causative agent of enteric redmouth disease in fish. *Vet Res, 46*, 103. doi:10.1186/s13567-015-0238-4

Kumar, S., Stecher, G., & Tamura, K. (2016). MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Mol Biol Evol, 33*(7), 1870-1874. doi:10.1093/molbev/msw054

Kyst.no (2020), https://www.kyst.no/article/slik-har-avlusningene-vaert-i-aar/

Larsen, A., Tao, Z., Bullard, S. A., & Arias, C. R. (2013). Diversity of the skin microbiota of fishes: evidence for host species specificity. *FEMS Microbiol Ecol, 85*(3), 483-494. doi:10.1111/1574-6941.12136

Lauzon, H., Gudmundsdottir S., Steinarsson A., Oddgeirsson, M., Martinsdottir, E., Gudmundsdottir B. (2010). Impact of probiotic intervention on microbial load and performance of Atlantic cod (*Gadus morhua L.*) juveniles, Aquaculture, Volume 310, Issues 1–2, 2010, Pages 139-144, ISSN 0044-8486, https://doi.org/10.1016/j.aquaculture.2010.10.017.

Lazado, C. C., & Caipang, C. M. (2014). Mucosal immunity and probiotics in fish. *Fish Shellfish Immunol, 39*(1), 78-89. doi:10.1016/j.fsi.2014.04.015

Lee, D. E., Lee, J., Kim, Y. M., Myeong, J. I., & Kim, K. H. (2016). Uncultured bacterial diversity in a seawater recirculating aquaculture system revealed by 16S rRNA gene amplicon sequencing. *J Microbiol, 54*(4), 296-304. doi:10.1007/s12275-016-5571-4

Lee, J., & Zhang, L. (2015). The hierarchy quorum sensing network in *Pseudomonas aeruginosa*. *Protein Cell, 6*(1), 26-41. doi:10.1007/s13238-014-0100-x

Li, Q., Ren, Y., & Fu, X. (2019). Inter-kingdom signaling between gut microbiota and their host. *Cell Mol Life Sci, 76*(12), 2383-2389. doi:10.1007/s00018-019-03076-7

Lilly, D. M., & Stillwell, R. H. (1965). Probiotics: growth-promoting factors produced by microorganisms. *Science, 147*(3659), 747-748. doi:10.1126/science.147.3659.747

Liu H., Guo, X., Gooneratne, R. Lai., R. Zeng,, C. Zhan, F., Wang W. The gut microbiome and degradation enzyme activity of wild freshwater fishes influenced by their tropic levelsSci. Rep., 6 (2016), 10.1038/srep24340

Lindell, K., Fahlgren, A., Hjerde, E., Willassen, N.-P., Fällman, M., Milton, D. L. (2012). Lipopolysaccharide O-antigen prevents phagocytosis of *Vibrio anguillarum* by rainbow trout (*Oncorhynchus mykiss*) skin epithelial cells PLoS One, 7(5):e37678. doi: 10.1371/journal.pone.0037678. Epub 2012 May 25.

Liu C. H., Chiu. H., Wang S. W., Cheng W. Dietary administration of the probiotic, *Bacillus subtilis* E20, enhances the growth, innate immune responses, and disease resistance of the grouper, *Epinephelus coioides* Fish Shellfish Immunol., 33 (2012), pp. 699-706

Llewellyn, M. S., Boutin, S., Hoseinifar, S. H., & Derome, N. (2014). Teleost microbiomes: the state of the art in their characterization, manipulation and importance in aquaculture and fisheries. *Frontiers in microbiology*, *5*, 207. https://doi.org/10.3389/fmicb.2014.00207

Llewellyn, M. S., Leadbeater, S., Garcia, C., Sylvain, F. E., Custodio, M., Ang, K. P., Derome, N. (2017). Parasitism perturbs the mucosal microbiome of Atlantic Salmon. *Sci Rep, 7*, 43465. doi:10.1038/srep43465

Llewellyn, M. S., McGinnity, P., Dionne, M., Letourneau, J., Thonier, F., Carvalho, G. R., Derome, N. (2016). The biogeography of the atlantic salmon (*Salmo salar*) gut microbiome. *Isme j, 10*(5), 1280-1284. doi:10.1038/ismej.2015.189

Lloyd-Price J, Abu-Ali G, Huttenhower C. (2016). The healthy human microbiome. Genome Med. 2016;8(1):51. doi:10.1186/s13073-016-0307-y

Loch, T. P., Scribner, K., Tempelman, R., Whelan, G., & Faisal, M. (2012). Bacterial infections of Chinook salmon, *Oncorhynchus tshawytscha* (Walbaum), returning to gamete collecting weirs in Michigan. *J Fish Dis, 35*(1), 39-50. doi:10.1111/j.1365-2761.2011.01322.x

Looft, T., Allen, H. K., Cantarel, B. L., Levine, U. Y., Bayles, D. O., Alt, D. P., Stanton, T. B. (2014). Bacteria, phages and pigs: the effects of in-feed antibiotics on the microbiome at different gut locations. *Isme j, 8*(8), 1566-1576. doi:10.1038/ismej.2014.12

Lopez, J. R., Pineiro-Vidal, M., Garcia-Lamas, N., de la Herran, R., Navas, J. I., Hachero-Cruzado, I., & Santos, Y. (2010). First isolation of *Tenacibaculum soleae* from diseased cultured wedge sole, *Dicologoglossa cuneata* (Moreau), and brill, *Scophthalmus rhombus* (L.). *J Fish Dis, 33*(3), 273-278. doi:10.1111/j.1365-2761.2009.01105.x

Lunder, T., Evensen, Ø., Holstad, G., & Håstein, T. (1995). "Winter ulcer" in the Atlantic salmon *Salmo salar*. Pathological and bacteriological investigations and transmission experiments. *Dis Aquat Organ, 23*(1), 39-49.

Løvoll, M., Wiik-Nielsen, C. R., Tunsjo, H. S., Colquhoun, D., Lunder, T., Sørum, H., & Grove, S. (2009). Atlantic salmon bath challenged with *Moritella viscosa*--pathogen invasion and host response. Fish Shellfish Immunol, 26(6), 877-884. doi:10.1016/j.fsi.2009.03.019

Marchesi, J. R., Adams, D. H., Fava, F., Hermes, G. D., Hirschfield, G. M., Hold, G., Hart, A. (2016). The gut microbiota and host health: a new clinical frontier. *Gut, 65*(2), 330-339. doi:10.1136/gutjnl-2015-309990

Martinez Cruz, P., Ibanez, A. L., Monroy Hermosillo, O. A., & Ramirez Saad, H. C. (2012). Use of probiotics in aquaculture. *ISRN Microbiol, 2012*, 916845. doi:10.5402/2012/916845

Mashburn, L. M., & Whiteley, M. (2005). Membrane vesicles traffic signals and facilitate group activities in a prokaryote. *Nature, 437*(7057), 422-425. doi:10.1038/nature03925

Masumura, K., & Wakabayashi, H. (1977). An Outbreak of Gliding Bacterial Disease in Hatchery-born Red Seabream (*Pagrus major*) and Gilthead (*Acanthopagrus schlegeli*) Fry in Hiroshima, Fish Pathology171-177,1977.12.

Mattilsynet (2014) Tap av laksefisk i sjø.

Mattilsynet (2019) Nasjonal tilsynskampanje 2018/2019, Velferd hos rensefisk

Maynard, C. L., Elson, C. O., Hatton, R. D., & Weaver, C. T. (2012). Reciprocal interactions of the intestinal microbiota and immune system. *Nature, 489*(7415), 231-241. doi:10.1038/nature11551

Mayville, P., Ji, G., Beavis, R., Yang, H., Goger, M., Novick, R. P., & Muir, T. W. (1999). Structure-activity analysis of synthetic autoinducing thiolactone peptides from *Staphylococcus aureus* responsible for virulence. *Proc Natl Acad Sci U S A, 96*(4), 1218-1223. doi:10.1073/pnas.96.4.1218

McFall-Ngai, M. J. (2014). The importance of microbes in animal development: lessons from the squid-vibrio symbiosis. *Annu Rev Microbiol, 68*, 177-194. doi:10.1146/annurev-micro-091313-103654

Mendelsohn, J. A. (2002). 'Like all that lives': biology, medicine and bacteria in the age of Pasteur and Koch. *Hist Philos Life Sci, 24*(1), 3-36.

Merrifield, D. L., Dimitroglou, A., Foey, A., Davies, S. J., Baker, R. T. M., Bøgwald, J., Ringø, E. (2010). The current status and future focus of probiotic and prebiotic applications for salmonids. *Aquaculture, 302*(1-2), 1-18. doi:10.1016/j.aquaculture.2010.02.007

Minich JJ, Poore GD, Jantawongsri K, Johnston C, Bowie K, Bowman J, Knight R, Nowak B, Allen EE. Microbial Ecology of Atlantic Salmon (*Salmo salar*) Hatcheries: Impacts of the Built Environment on Fish Mucosal Microbiota. Appl Environ Microbiol. 2020 Jun 2;86(12):e00411-20. doi: 10.1128/AEM.00411-20. PMID: 32303543; PMCID: PMC7267192.

Ministero della Salute, Commissione unica per la nutrizione e la dietetica. Guidelines on probiotics and prebiotics. Ministero della Salute [online], http://www.salute.gov.it/imgs/C_17_pubblicazioni_1016_allegato.pdf (2013).

Mohapatra, S., Chakraborty, T., Kumar, V., DeBoeck, G., & Mohanta, K. N. (2013). Aquaculture and stress management: a review of probiotic intervention. *J Anim Physiol Anim Nutr (Berl), 97*(3), 405-430. doi:10.1111/j.1439-0396.2012.01301.x

Molina, J. P., Santamaría, A., Luna, A., F. Martínez-Díaz, S., & Rojas, M. (2009). Effect of potential probiotic bacteria on growth and survival of tilapia *Oreochromis niloticus L.*, cultured in the laboratory under high density and suboptimum temperature. *Aquaculture Research, 40*, 887-894. doi:10.1111/j.1365-2109.2009.02172.x

Mongeau, J. D. (1981). Hagan and Bruner's Infectious Diseases of Domestic Animals, 7th Edition. *The Canadian Veterinary Journal, 22*(12), 371-371.

Moran, P. G., Al-Hebshi, N. (2020) Editorial: The Human Microbiome and Cancer, *Front. Microbiol*., 21 July 2020 | https://doi.org/10.3389/fmicb.2020.01514

Moreno-Andres, J., Acevedo-Merino, A., & Nebot, E. (2018). Study of marine bacteria inactivation by photochemical processes: disinfection kinetics and growth modeling after treatment. *Environ Sci Pollut Res Int, 25*(28), 27693-27703. doi:10.1007/s11356-017-1185-6

Moriano-Gutierrez, S., Koch, E. J., Bussan, H., Romano, K., Belcaid, M., Rey, F. E., McFall-Ngai, M. J. (2019). Critical symbiont signals drive both local and systemic changes in diel and developmental host gene expression. *Proc Natl Acad Sci U S A, 116*(16), 7990-7999. doi:10.1073/pnas.1819897116

Morris, J. J. (2018). What is the hologenome concept of evolution? *F1000Res, 7*. doi:10.12688/f1000research.14385.1

Mukherjee, S., & Bassler, B. L. (2019). Bacterial quorum sensing in complex and dynamically changing environments. *Nat Rev Microbiol*. doi:10.1038/s41579-019-0186-5

Nayak, S. K. (2010). Probiotics and immunity: a fish perspective. *Fish Shellfish Immunol, 29*(1), 2-14. doi:10.1016/j.fsi.2010.02.017

Nealson, K. H., Platt, T., & Hastings, J. W. (1970). Cellular control of the synthesis and activity of the bacterial luminescent system. *J Bacteriol, 104*(1), 313-322.

Nelson KE. (2013). Microbiomes. Microb Ecol. 2013;65(4):916-919. doi:10.1007/s00248-013-0227-y

Nematollahi, A., Decostere, A., Pasmans, F., & Haesebrouck, F. (2003). *Flavobacterium psychrophilum* infections in salmonid fish. *J Fish Dis, 26*(10), 563-574. doi:10.1046/j.1365-2761.2003.00488.x

Newaj-Fyzul, Aweeda & Al-Harbi, Ahmed & Austin, Brian. (2014). Review: Developments in the use of probiotics for disease control in aquaculture. Aquaculture. 431. 1-11. 10.1016/j.aquaculture.2013.08.026.

NilsenA., Viljugren H., Røsæg M. V., Colquhon D. (2014). Rensefiskhelse-kartlegging av dødelighetssårsaker. Veterinærinstituttets rapportserie 12-2014.

Norwegian fisheries directorate, (2018). https://www.fiskeridir.no/Akvakultur/Tall-og analyse/Loennsomhetsundersoekelse-for-laks-og-et regnbueoerret/Matfiskproduksjon-laks-og-regnbueoerret

Norwegian fisheries directorate, (2019). Norwegian fisheries directorate. (https://www.fiskeridir.no/Akvakultur/Tall-og-analyse/Akvakulturstatistikk-tidsserier/Laks-regnbueoerret-og-oerret/Matfiskproduksjon)

Nærings- og fiskeridepartementet, (2017). Forskrift om tiltak for å forebygge, begrense og bekjempe pankreassykdom (PD) hos akvakulturdyr. https://lovdata.no/dokument/SF/forskrift/2017-08-29-1318?q=PD

O'Byrne-Ring, N., Dowling, K., Cotter, D., Whelan, K., & MacEvilly, U. (2003). Changes in mucus cell numbers in the epidermis of the Atlantic salmon at the onset of smoltification. *63*(6), 1625-1630. doi:10.1111/j.1095-8649.2003.00273.x

Oh, H. S., Tan, C. H., Low, J. H., Rzechowicz, M., Siddiqui, M. F., Winters, H., Rice, S. A. (2017). Quorum quenching bacteria can be used to inhibit the biofouling of reverse osmosis membranes. *Water Res, 112*, 29-37. doi:10.1016/j.watres.2017.01.028

Okada, M., Sato, I., Cho, S. J., Iwata, H., Nishio, T., Dubnau, D., & Sakagami, Y. (2005). Structure of the Bacillus subtilis quorum-sensing peptide pheromone ComX. *Nat Chem Biol, 1*(1), 23-24. doi:10.1038/nchembio709

Olafsen, J. A. (2001). Interaction between fish larvae and bacteria in marine aquaculture. *Aquculture,* 200, 223-247. https://doi.org/10.1016/S0044-8486(01)00702-5

Olsen, A. B., Gulla, S., Steinum, T., Colquhoun, D. J., Nilsen, H. K., & Duchaud, E. (2017). Multilocus sequence analysis reveals extensive genetic variety within *Tenacibaculum* spp. associated with ulcers in sea-farmed fish in Norway. *Vet Microbiol, 205*, 39-45. doi:10.1016/j.vetmic.2017.04.028

Olsen, A. B., Nilsen, H., Sandlund, N., Mikkelsen, H., Sørum, H., & Colquhoun, D. J. (2011). *Tenacibaculum* sp. associated with winter ulcers in sea-reared Atlantic salmon *Salmo salar*. *Dis Aquat Organ, 94*(3), 189-199. doi:10.3354/dao02324

Osland, E. (1990). Bruke havet – pionértid i norsk fiskeoppdrett

Ozen, M., & Dinleyici, E. C. (2015). The history of probiotics: the untold story. *Benef Microbes, 6*(2), 159-165. doi:10.3920/bm2014.0103

Pacheco, A. R., & Sperandio, V. (2009). Inter-kingdom signaling: chemical language between bacteria and host. *Curr Opin Microbiol, 12*(2), 192-198. doi:10.1016/j.mib.2009.01.006

Patent US (2020). Probiotic bacteria for fish, US 2020 236 975 A1. https://patentswarm.com/patents/US20200236975A1

Pesci, E. C., Milbank, J. B., Pearson, J. P., McKnight, S., Kende, A. S., Greenberg, E. P., & Iglewski, B. H. (1999). Quinolone signaling in the cell-to-cell communication system of *Pseudomonas aeruginosa. Proc Natl Acad Sci U S A, 96*(20), 11229-11234. doi:10.1073/pnas.96.20.11229

Pezzulo AA, Kelly PH, Nassar BS, et al. (2013).Abundant DNase I-sensitive bacterial DNA in healthy porcine lungs and its implications for the lung microbiome. Appl Environ Microbiol. 2013;79(19):5936-5941. doi:10.1128/AEM.01752-13

Pharmaq Analytic (2019) https://www.pharmaq-analytiq.com/kunnskap/rensefisk/kratesyke-hos-rognkjeks/

Pineiro-Vidal, M., Gijon, D., Zarza, C., & Santos, Y. (2012). *Tenacibaculum dicentrarchi* sp. nov., a marine bacterium of the family *Flavobacteriaceae* isolated from European sea bass. *Int J Syst Evol Microbiol, 62*(Pt 2), 425-429. doi:10.1099/ijs.0.025122-0

Popoff, M. R. (2018). "Bacterial Toxins" Section in the Journal Toxins: A Fantastic Multidisciplinary Interplay between Bacterial Pathogenicity Mechanisms, Physiological Processes, Genomic Evolution, and Subsequent Development of Identification Methods, Efficient Treatment, and Prevention of Toxigenic Bacteria. *Toxins (Basel), 10*(1). doi:10.3390/toxins10010044

Poppe, T. (2017). Norsk veterinærtidsskrift nr. 4, s 214-218

Powell, M., Carson, J., & van Gelderen, R. (2004). Experimental induction of gill disease in Atlantic salmon *Salmo salar* smolts with *Tenacibaculum maritimum. Dis Aquat Organ, 61*(3), 179-185. doi:10.3354/dao061179

Rader, B. A., & Nyholm, S. V. (2012). Host/microbe interactions revealed through "omics" in the symbiosis between the Hawaiian bobtail squid *Euprymna scolopes* and the bioluminescent bacterium *Vibrio fischeri. Biol Bull, 223*(1), 103-111. doi:10.1086/BBLv223n1p103

Ramirez, C., & Romero, J. (2017). The Microbiome of Seriola lalandi of Wild and Aquaculture Origin Reveals Differences in Composition and Potential Function. *Front Microbiol, 8*, 1844. doi:10.3389/fmicb.2017.01844

Rawls, J. F., Mahowald, M. A., Ley, R. E., & Gordon, J. I. (2006). Reciprocal gut microbiota transplants from zebrafish and mice to germ-free recipients reveal host habitat selection. *Cell, 127*(2), 423-433. doi:10.1016/j.cell.2006.08.043

Read, A. (2013). An Introduction to Marine Mammal Biology and Conservation by E. C. M. Parsons (Vol. 29).

Reddy, S. (2015). Probiotics in aquaculture: importance, influence and future perspectives. *International Journal of Bioassays, 4*, 3710-3718.

Reed, G. B., & Spence, C. M. (1929). The intestinal and slime flora of the haddock: a preliminary report. *Contributions to Canadian Biology and Fisheries, 4*(1), 257-264. doi:10.1139/f29-019

Rehman, Z. U., & Leiknes, T. (2018). Quorum-Quenching Bacteria Isolated From Red Sea Sediments Reduce Biofilm Formation by *Pseudomonas aeruginosa*. *Front Microbiol, 9*, 1354. doi:10.3389/fmicb.2018.01354

Ringø, E., Salinas, I., Olsen, R. E., Nyhaug, A., Myklebust, R. & Mayhew, T. M. (2007). Histological changes in intestine of Atlantic salmon (*Salmo salar L.*) following in vitro exposure to pathogenic and probiotic bacterial strains. *Cell and Tissue Research, 328*(1), 109-116. doi:10.1007/s00441-006-0323-0

Ringø, E., Strøm, E., & Tabachek, J.-A. (1995). Intestinal microflora of salmonids: a review. *26*(10), 773-789. doi:10.1111/j.1365-2109.1995.tb00870.x

Roalkvam, I., Dronen, K., Dahle, H., & Wergeland, H. I. (2019). Microbial Communities in a Flow-Through Fish Farm for Lumpfish (*Cyclopterus lumpus L.*) During Healthy Rearing Conditions. *Front Microbiol, 10*, 1594. doi:10.3389/fmicb.2019.01594

Roeselers, G., Mittge, E. K., Stephens, W. Z., Parichy, D. M., Cavanaugh, C. M., Guillemin, K., & Rawls, J. F. (2011). Evidence for a core gut microbiota in the zebrafish. *Isme j, 5*(10), 1595-1608. doi:10.1038/ismej.2011.38

Rombout J.,Yang G., Kiron V. (2014). Adaptive immune responses at mucosal surfaces of teleost fish, Fish & Shellfish Immunology, Volume 40, Issue 2, 2014, Pages 634-643,ISSN 1050-4648, https://doi.org/10.1016/j.fsi.2014.08.020.

Romero, Manuel & Muras, Andrea & Mayer, Celia & Bujan, Noemi & Magarinos, Beatriz & Otero, Ana. (2014). In vitro quenching of fish pathogen Edwardsiella tarda AHL production using marine bacterium *Tenacibaculum sp* strain 20J cell extracts. Diseases of aquatic organisms. 108. 217-25. 10.3354/dao02697.

Romero, R., Gomez-Lopez, N., Winters, Andrew D., Jung, E., Shaman, M., Bieda, J., Panaitescu, B., Pacora, P., Erez, O., Greenberg, J. M., Ahmad, M. M., Hsu, C. and Theis, K. R.. "Evidence that intra-amniotic infections are often the result of an ascending invasion – a molecular microbiological study" Journal of Perinatal Medicine, vol. 47, no. 9, 2019, pp. 915-931. https://doi.org/10.1515/jpm-2019-0297

Romstad, A. B., Reitan, L. J., Midtlyng, P., Gravningen, K., & Evensen, O. (2013). Antibody responses correlate with antigen dose and in vivo protection for oil-adjuvanted, experimental furunculosis (*Aeromonas salmonicida subsp. salmonicida*) vaccines in Atlantic salmon (*Salmo salar L.*) and can be used for batch potency testing of vaccines. *Vaccine, 31*(5), 791-796. doi:10.1016/j.vaccine.2012.11.069

Rosenberg, E., & Zilber-Rosenberg, I. (2011). Symbiosis and development: the hologenome concept. *Birth Defects Res C Embryo Today, 93*(1), 56-66. doi:10.1002/bdrc.20196

Rosenberg, E., & Zilber-Rosenberg, I. (2018). The hologenome concept of evolution after 10 years. *Microbiome, 6*(1), 78. doi:10.1186/s40168-018-0457-9

Rosenberg, E., & Zilber-Rosenberg, I. (2019). The Hologenome Concept of Evolution: Medical Implications. *Rambam Maimonides Med J, 10*(1). doi:10.5041/rmmj.10359

Ross, A. A., Rodrigues Hoffmann, A., & Neufeld, J. D. (2019). The skin microbiome of vertebrates. *Microbiome, 7*(1), 79. doi:10.1186/s40168-019-0694-6

Ross, L. N., & Woodward, J. F. (2016). Koch's postulates: An interventionist perspective. *Stud Hist Philos Biol Biomed Sci, 59*, 35-46. doi:10.1016/j.shpsc.2016.06.001

Rusnock, A. (2009). Catching cowpox: the early spread of smallpox vaccination, 1798-1810. *Bull Hist Med, 83*(1), 17-36. doi:10.1353/bhm.0.0160

Saitou, N., & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol, 4*(4), 406-425. doi:10.1093/oxfordjournals.molbev.a040454

Salinas, I., Myklebust, R., Esteban, M. A., Olsen, R. E., Meseguer, J., & Ringo, E. (2008). In vitro studies of *Lactobacillus delbrueckii subsp lactis* in Atlantic salmon (*Salmo salar L.*) foregut: Tissue responses and evidence of protection against *Aeromonas salmonicida subsp salmonicida* epithelial damage. *Veterinary Microbiology, 128*(1-2), 167-177. doi:10.1016/j.vetmic.2007.10.011

Sanders, J. G., Lukasik, P., Frederickson, M. E., Russell, J. A., Koga, R., Knight, R., & Pierce, N. E. (2017). Dramatic Differences in Gut Bacterial Densities Correlate with Diet and Habitat in Rainforest Ants. *Integr Comp Biol, 57*(4), 705-722. doi:10.1093/icb/icx088

Sarrafzadeh, A. S., Sarafian, N., von Gladiss, A., Unterberg, A. W., & Lanksch, W. R. (2001). Ibn Sina (Avicenna). Historical note. *Neurosurg Focus, 11*(2), E5.

Schauder, S., Shokat, K., Surette, M. G., & Bassler, B. L. (2001). The LuxS family of bacterial auto-inducers: biosynthesis of a novel quorum-sensing signal molecule. *Mol Microbiol, 41*(2), 463-476.

Schmidt, V., Amaral-Zettler, L., Davidson, J., Summerfelt, S., & Good, C. (2016). Influence of Fishmeal-Free Diets on Microbial Communities in Atlantic Salmon (*Salmo salar*) Recirculation Aquaculture Systems. *Appl Environ Microbiol, 82*(15), 4470-4481. doi:10.1128/aem.00902-16

Schmidt, V. T., Smith, K. F., Melvin, D. W., & Amaral-Zettler, L. A. (2015). Community assembly of a euryhaline fish microbiome during salinity acclimation. *Mol Ecol, 24*(10), 2537-2550. doi:10.1111/mec.13177

Schrijver, R & Ollevier, Frans. (2000). Protein digestion in juvenile turbot (*Scophthalmus maximus*) and effects of dietary administration of *Vibrio proteolyticus*. Aquaculture. 186. 107-116. 10.1016/S0044-8486(99)00372-5.

Schøning, G. (1775). Hans Reise giennom en Deel af Norge i de Aar 1773, 1774, 1775.

Sekirov, I., Russell, S. L., Antunes, L. C., & Finlay, B. B. (2010). Gut microbiota in health and disease. *Physiol Rev, 90*(3), 859-904. doi:10.1152/physrev.00045.2009

Semova, I., Carten, J. D., Stombaugh, J., Mackey, L. C., Knight, R., Farber, S. A., & Rawls, J. F. (2012). Microbiota regulate intestinal absorption and metabolism of fatty acids in the zebrafish. *Cell Host Microbe, 12*(3), 277-288. doi:10.1016/j.chom.2012.08.003

Smith, J. N., & Ahmer, B. M. (2003). Detection of other microbial species by Salmonella: expression of the SdiA regulon. J Bacteriol, 185(4), 1357-1366. doi:10.1128/jb.185.4.1357-1366.2003

Småge, S. B., Brevik, O. J., Duesund, H., Ottem, K. F., Watanabe, K., & Nylund, A. (2016). *Tenacibaculum finnmarkense* sp. nov., a fish pathogenic bacterium of the family Flavobacteriaceae isolated from Atlantic salmon. *Antonie Van Leeuwenhoek, 109*(2), 273-285. doi:10.1007/s10482-015-0630-0

Småge, S. B., Frisch, K., Brevik, O. J., Watanabe, K., & Nylund, A. (2016). First isolation, identification and characterisation of *Tenacibaculum maritimumin* Norway, isolated fromdiseased farmed sea lice cleaner fish *Cyclopterus lumpus L*. *Aquaculture, 464*, 178-184. doi:10.1016/j.aquaculture.2016.06.030

Småge, S. B., Frisch, K., Vold, V., Duesund, H., Brevik, Ø. J., Olsen, R. H., Nylund, A. (2018). Induction of tenacibaculosis in Atlantic salmon smolts using *Tenacibaculum finnmarkense* and the evaluation of a whole cell inactivated vaccine. *Aquaculture, 495*, 858-864. doi:https://doi.org/10.1016/j.aquaculture.2018.06.063

Sommerset, I., Bang Jensen, B., Bornø, B., Haukaas, A. og Brun, E. Fiskehelserapporten 2020 (2021), ISSN 1890-3290

Speare DJ, MacNair N, Hammell KL. (1995).Demonstration of tank effect on growth indices of juvenile rainbow trout (*Oncorhynchus mykiss*) during an ad libitum feeding trial. Am J Vet Res. 1995 Oct;56(10):1372-9. PMID: 8928957.

Steinum, T., Sjåstad, K., Falk, K., Kvellestad, A., & Colquhoun, D. J. (2009). An RT PCR-DGGE survey of gill-associated bacteria in Norwegian seawater-reared Atlantic salmon suffering proliferative gill inflammation. *Aquaculture, 293*(3), 172-179. doi:https://doi.org/10.1016/j.aquaculture.2009.05.006

Steinum, T. M., Karatas, S., Martinussen, N. T., Meirelles, P. M., Thompson, F. L., & Colquhoun, D. J. (2016). Multilocus Sequence Analysis of Close Relatives Vibrio anguillarum and Vibrio ordalii. *Appl Environ Microbiol, 82*(18), 5496-5504. doi:10.1128/aem.00620-16

Stickney, R.R. (2009). Aquaculture 2nd ed. (Wallingford, UK:CABI:2009)

Suez, J., Zmora, N., Zilberman-Schapira, G., Mor, U., Dori-Bachash, M., Bashiardes, S., Elinav, E. (2018). Post-Antibiotic Gut Mucosal Microbiome Reconstitution Is Impaired by Probiotics and Improved by Autologous FMT. *Cell, 174*(6), 1406-1423.e1416. doi:10.1016/j.cell.2018.08.047

Sullam, K. E., Essinger, S. D., Lozupone, C. A., O'Connor, M. P., Rosen, G. L., Knight, R., Russell, J. A. (2012). Environmental and ecological factors that shape the gut bacterial communities of fish: a meta-analysis. *Mol Ecol, 21*(13), 3363-3378. doi:10.1111/j.1365-294X.2012.05552.x

Suzuki, M., Nakagawa, Y., Harayama, S., & Yamamoto, S. (2001). Phylogenetic analysis and taxonomic study of marine Cytophaga-like bacteria: proposal for Tenacibaculum gen. nov. with *Tenacibaculum maritimum* comb. nov. and *Tenacibaculum ovolyticum* comb. nov., and description of *Tenacibaculum mesophilum* sp. nov. and *Tenacibaculum amylolyticum* sp. nov. *Int J Syst Evol Microbiol, 51*(Pt 5), 1639-1652. doi:10.1099/00207713-51-5-1639

Tadiso T., Sharma A., Hordvik I. (2001) Analysis of polymeric immunoglobulin receptor- and CD300-like molecules from Atlantic salmon,Molecular Immunology,Volume 49, Issue 3,2011,Pages 462-473,ISSN 0161-5890,https://doi.org/10.1016/j.molimm.2011.09.013.

Taoka, Y., Maeda, H., Jo, JY. *et al.* Growth, stress tolerance and non-specific immune response of Japanese flounder *Paralichthys olivaceus* to probiotics in a closed recirculating system. *Fish Sci* 72, 310–321 (2006). https://doi.org/10.1111/j.1444-2906.2006.01152.x

Takle, H. R., Ytteborg, E., Nielsen, K. V., Karlsen, C. R., Nilsen, H. K., Sveen, L., Nilsen, A. (2015). Sårproblematikk og hudhelse i laks-og regnbueørrettoppdrett.

Tamura, K., Nei, M., & Kumar, S. (2004). Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proc Natl Acad Sci U S A, 101*(30), 11030-11035. doi:10.1073/pnas.0404206101

Tapia-Paniagua, S., Lobo, C., Moreno-Ventas, X., de la Banda, I. G., Morinigo, M. A., & Balebona, M. C. (2014). Probiotic supplementation influences the diversity of the intestinal microbiota during early stages of farmed senegalese sole (*Solea Senegalensis*, Kaup 1858). *Mar Biotechnol (NY), 16*(6), 716-728. doi:10.1007/s10126-014-9588-6

Tilseth, S., Hansen, T., & Møller, D. (1991). *Historical development of salmon culture* (Vol. 98).

Tomasz, A. (1965). Control of the competent state in *Pneumococcus* by a hormone-like cell product: an example for a new type of regulatory mechanism in bacteria. *Nature, 208*(5006), 155-159.

Tomasz, A. (1966). Model for the mechanism controlling the expression of competent state in Pneumococcus cultures. *J Bacteriol, 91*(3), 1050-1061.

Tuan, Tran & Pham Minh, Duc & Hatai, Kishio. (2013). Overview of the use of probiotics in aquaculture. International Journal of Research in Fisheries and Aquaculture. 3. 89-97.

United nations (2017) World population prospects. The 2017 Revision, key Findings and advanced tables. Population Division, United Nations, New York, NY.

Urbanczyk, H., Ast, J. C., Higgins, M. J., Carson, J., & Dunlap, P. V. (2007). Reclassification of V*ibrio fischeri, Vibrio logei, Vibrio salmonicida* and V*ibrio wodanis* as *Aliivibrio fischeri* gen. nov., comb. nov., *Aliivibrio logei* comb. nov., *Aliivibrio salmonicida* comb. nov. and *Aliivibrio wodanis* comb. nov. *Int J Syst Evol Microbiol, 57*(Pt 12), 2823-2829. doi:10.1099/ijs.0.65081-0

Uzman, A. (2003). Molecular biology of the cell (4th ed.): Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., and Walter, P (Vol. 31).

Vadstein, O., Attramadal, K. J. K., Bakke, I., & Olsen, Y. (2018). K-Selection as Microbial Community Management Strategy: A Method for Improved Viability of Larvae in Aquaculture. *Front Microbiol, 9*, 2730. doi:10.3389/fmicb.2018.02730

van der Marel, M., Caspari, N., Neuhaus, H., Meyer, W., Enss, M. L., & Steinhagen, D. (2010). Changes in skin mucus of common carp, *Cyprinus carpio* L., after exposure to water with a high bacterial load. *J Fish Dis, 33*(5), 431-439. doi:10.1111/j.1365-2761.2010.01140.x

van Gelderen, R., Carson, J., Gudkovs, N., & Nowak, B. (2010). Physical characterisation of *Tenacibaculum maritimum* for vaccine development. *J Appl Microbiol, 109*(5), 1668-1676. doi:10.1111/j.1365-2672.2010.04795.x

van Gelderen, R., Carson, J., & Nowak, B. (2010). Experimentally induced marine flexibacteriosis in Atlantic salmon smolts *Salmo salar*. I. Pathogenicity. *Dis Aquat Organ, 91*(2), 121-128. doi:10.3354/dao02219

van Gelderen, R., Carson, J., & Nowak, B. (2011). Experimentally induced marine flexibacteriosis in Atlantic salmon smolts *Salmo salar*. II. Pathology. *Dis Aquat Organ, 95*(2), 125-135. doi:10.3354/dao02329

Vaxxinova, (2019). sphttps://www.vaxxinova.no/tjenester/autogene-vaksiner/vaksinering-av-rognkjeks-cyclopterus-lumpus/.

Verschuere, L., Rombaut, G., Sorgeloos, P., & Verstraete, W. (2000). Probiotic bacteria as biological control agents in aquaculture. *Microbiol Mol Biol Rev, 64*(4), 655-671.

Veterinæristituttet, https://www.vetinst.no/fagomrader/desinfeksjon/veileder-metoder-godkjent-for-desinfeksjon-av-vann-til-fra-akvakulturrelatert-virksomhet

Veterinærinstituttet (2019), https://www.vetinst.no/nyheter/pasteurella-kan-gi-sykdom-hos-bade-laks-og-rognkjeks

Veterinærinstituttet (2020). Kontali Produktivitetskonferanse 2020. https://my.kontali.no/3-eksperter-paa-under-1-time/

Villamil L., Figueras A., Planas M., Novoa B. (2010), Pediococcus acidilactici in the culture of turbot (*Psetta maxima*) larvae: Administration pathways, Aquaculture, Volume 307, Issues 1–2, 2010, Pages 83-88, ISSN 0044-8486 https://doi.org/10.1016/j.aquaculture.2010.07.004.

Walter, C. W. (1976). Letter: Handwashing and Semmelweiss. *Ann Intern Med, 85*(3), 398.

Walter, J., & Ley, R. (2011). The human gut microbiome: ecology and recent evolutionary changes. *Annu Rev Microbiol, 65*, 411-429. doi:10.1146/annurev-micro-090110-102830

Wang, J., Wang, C., Yu, H., Dela Ahator, S., Wu, X., Lv, S., & Zhang, L. H. (2019). Bacterial quorum sensing signal IQS induces host cell apoptosis by targeting POT1-p53 signalling pathway. *Cell Microbiol*, e13076. doi:10.1111/cmi.13076

Whiteley, M., Diggle, S. P., & Greenberg, E. P. (2017). Progress in and promise of bacterial quorum sensing research. *Nature, 551*(7680), 313-320. doi:10.1038/nature24624

Wong, S., Waldrop, T., Summerfelt, S., Davidson, J., Barrows, F., Kenney, P. B., Good, C. (2013). Aquacultured rainbow trout (*Oncorhynchus mykiss*) possess a large core intestinal microbiota that is resistant to variation in diet and rearing density. *Appl Environ Microbiol, 79*(16), 4974-4984. doi:10.1128/aem.00924-13

World Bank (2013) Fish to 2030: Prospects for Fisheries and Aquaculture. The World bank, Washington DC.

Wynne JW, Thakur KK, Slinger J, Samsing F, Milligan B, Powell JFF, McKinnon A, Nekouei O, New D, Richmond Z, Gardner I and Siah A (2020) Microbiome Profiling Reveals a Microbial Dysbiosis During a Natural Outbreak of Tenacibaculosis (Yellow Mouth) in Atlantic Salmon. Front. Microbiol. 11:586387. doi: 10.3389/fmicb.2020.586387

Xavier, K. B., & Bassler, B. L. (2005a). Interference with AI-2-mediated bacterial cell-cell communication. *Nature, 437*(7059), 750-753. doi:10.1038/nature03960

Xavier, K. B., & Bassler, B. L. (2005b). Regulation of uptake and processing of the quorum-sensing autoinducer AI-2 in E. coli. *J Bacteriol, 187*(1), 238-248. doi:10.1128/jb.187.1.238-248.2005

Yoshimizu, M., Kamiyama, K., Kimura, T., & Sakai, M. (1976). Studies on intestinal microflora of salmonids.4. intestinal microflora of fresh-water salmon. *Bulletin of the Japanese Society of Scientific Fisheries, 42*(11), 1281-1290.

Yoshimizu, M., Kimura, T., & Sakai, M. (1976a). Studies of intestinal microflora of salmonids. 2. Effects of artificial transplanting from fresh-water into sea-water on intestinal microflora of feeding and non-feeding fish. *Bulletin of the Japanese Society of Scientific Fisheries, 42*(8), 863-873.

Yoshimizu, M., Kimura, T., & Sakai, M. (1976b). Studies on intestinal microflora of salmonids.1. Intestinal microflora of fish reared in fresh-water and sea-water. *Bulletin of the Japanese Society of Scientific Fisheries, 42*(1), 91-99.

Yoshimizu, M., Kimura, T., & Sakai, M. (1976c). Studies on intestinal microflora of salmonids. 3. Intestinal microflora of salmon living in open sea. *Bulletin of the Japanese Society of Scientific Fisheries, 42*(8), 875-884.

Yoshimizu, M., Kimura, T., & Sakai, M. (1976d). Studies on intestinal microflora of salmonids. 5. Intestinal microflora of anadromous salmon. *Bulletin of the Japanese Society of Scientific Fisheries, 42*(11), 1291-1298.

Zaineldin, A. I., Hegazi, S., Koshio, S., Ishikawa, M., Bakr, A., El-Keredy, A. M. S., Yukun, Z. (2018). *Bacillus subtilis* as probiotic candidate for red sea bream: Growth performance, oxidative status, and immune response traits. *Fish Shellfish Immunol, 79*, 303-312. doi:10.1016/j.fsi.2018.05.035

Zanuzzo FS, Beemelmanns A, Hall JR, Rise ML, Gamperl AK. The Innate Immune Response of Atlantic Salmon (*Salmo salar*) Is Not Negatively Affected by High Temperature and Moderate Hypoxia. Front Immunol. 2020;11:1009. Published 2020 May 27. doi:10.3389/fimmu.2020.01009

Zarkasi, K. Z., Abell, G. C., Taylor, R. S., Neuman, C., Hatje, E., Tamplin, M. L., Bowman, J. P. (2014). Pyrosequencing-based characterization of gastrointestinal bacteria of Atlantic salmon (*Salmo salar L.*) within a commercial mariculture system. *J Appl Microbiol, 117*(1), 18-27. doi:10.1111/jam.12514

Zarkasi, K. Z., Taylor, R. S., Abell, G. C., Tamplin, M. L., Glencross, B. D., & Bowman, J. P. (2016). Atlantic Salmon (*Salmo salar L.*) Gastrointestinal Microbial Community Dynamics in Relation to Digesta Properties and Diet. *Microb Ecol, 71*(3), 589-603. doi:10.1007/s00248-015-0728-y

Zarkasi, K. Z., Taylor, R. S., Glencross, B. D., Abell, G. C. J., Tamplin, M. L., & Bowman, J. P. (2017). In vitro characteristics of an Atlantic salmon (*Salmo salar L.*) hind gut microbial community in relation to different dietary treatments. *Res Microbiol, 168*(8), 751-759. doi:10.1016/j.resmic.2017.07.003

Zhang, C., Parrello, D., Brown, P. J. B., Wall, J. D., & Hu, Z. (2018). A novel whole-cell biosensor of *Pseudomonas aeruginosa* to monitor the expression of quorum sensing genes. *Appl Microbiol Biotechnol, 102*(14), 6023-6038. doi:10.1007/s00253-018-9044-z

Zhou, Z., Yao, B., Romero, J., Waines, P., Ringø, E., Emery, M., L. Merrifield, D. (2014). Methodological Approaches Used to Assess Fish Gastrointestinal Communities. In (pp. 101-127).

Zilber-Rosenberg, I., & Rosenberg, E. (2008). Role of microorganisms in the evolution of animals and plants: the hologenome theory of evolution. *FEMS Microbiol Rev, 32*(5), 723-735. doi:10.1111/j.1574-6976.2008.00123.x

Øen, O., E. (1996). Hvalfangerklubben, avskrift av et radioforedrag. http://www.hvalfangerklubben.net/index.php/artikler?id=131

8. SCIENTIFIC PAPERS I-III

I

Received: 22 November 2018 | Revised: 1 February 2019 | Accepted: 2 February 2019

DOI: 10.1111/jfd.12982

ORIGINAL ARTICLE

WILEY Journal of Fish Diseases

An outbreak of acute disease and mortality in Atlantic salmon (*Salmo salar*) post-smolts in Norway caused by *Tenacibaculum dicentrarchi*

Øystein Klakegg[1,2] | Takele Abayneh[3] | Aud Kari Fauske[1,2] | Michael Fülberth[4] | Henning Sørum[2]

[1]Previwo AS, Oslo, Norway

[2]Department of Food Safety and Infection Biology, Faculty of Veterinary Medicine, Norwegian University of Life Sciences, Oslo, Norway

[3]National Veterinary Institute, Bishoftu/Debre-zeit, Ethiopia

[4]Njord Salmon AS, Kjørsvikbugen, Norway

Correspondence
Øystein Klakegg, Previwo AS, Oslo, Norway.
Emails: oystein.klakegg@previwo.no; post@previwo.no

Funding information
Norges Forskningsråd, Grant/Award Number: 260204/O30; Norwegian Research Council, Grant/Award Number: 260204 and O30

Abstract

An outbreak of disease characterized by skin ulcers, fin rot and mortality was observed a few days after the transfer of Atlantic salmon (*Salmo salar*) from a freshwater smolt production facility to a land-based seawater post-smolt site. Dead and moribund fish had severe skin and muscle ulcers, often 2–6 cm wide, particularly caudal to the pectoral fins. Microscopic examination of smears from ulcers and head kidney identified long, slender Gram-negative rods. Histopathological analysis revealed abundance of long, slender *Tenacibaculum*-like bacteria in ulcers and affected fins. Genetic characterization using multilocus sequence analysis (MLSA) of seven housekeeping genes, including *atpA*, *dnaK*, *glyA*, *gyrB*, *infB*, *rlmN* and *tgt*, revealed that the isolates obtained during the outbreak were all clustered with the *Tenacibaculum dicentrarchi*-type strain (USC39/09^T) from Spain. Two bath challenge experiments with Atlantic salmon and an isolate of *T. dicentrarchi* from the outbreak were performed. No disease or mortality was observed in the first trial. In the second trial with a higher challenge dose of *T. dicentrarchi* and longer challenge time, we got 100% mortality within 48 hr. This is the first reported outbreak of disease caused by *T. dicentrarchi* in Norwegian farmed Atlantic salmon.

KEYWORDS
Atlantic salmon, disease, Norway, post-smolt, *Tenacibaculum dicentrarchi*

1 | INTRODUCTION

The establishment of land-based post-smolt aquaculture of Atlantic salmon (*Salmo salar*) is increasing particularly due to the challenges caused by sea lice (*Lepeophtheirus salmonis*) at sea sites. In tanks at the post-smolt sites, the salmon can grow larger without being exposed to sea lice, and thus, the time of exposure to sea lice becomes shorter when salmon launched to open net cages at sea. Unfortunately, it has been observed that ulcers and increased mortality is a challenge at post-smolt sites (Institute of Marine Research, 2018).

Bacteria in the genus *Tenacibaculum* are Gram-negative, thread-formed, 1.5- to 30-µm-long, yellow-pigment-producing bacteria that are members of the family Flavobacteriaceae. The term "Tenax" means adhere, and therefore, the genus "*Tenacibaculum*" refers to a rod-shaped bacterium that adheres to the surfaces of marine organisms. The species are all isolated from marine environment and grow on media containing sea water (Suzuki, Nakagawa, Harayama, & Yamamoto, 2001).

The number of species in genus *Tenacibaculum* is rapidly increasing, and so far, 28 species have been validly published including *T. maritimum*^{T}, *T. adriaticum*, *T. aestuarii*, *T. aestuariivivum*,

T. agarivorans, *T. aiptasiae*, *T. amylolyticum*, *T. ascidiaceicola*, *T. caenipelagi*, *T. crassostreae*, *T. dicentrarchi*, *T. discolor*, *T. gallaicum*, *T. geojense*, *T. haliotis*, *T. holothuriorum*, *T. insulae*, *T. jejujense*, *T. litopenaei*, *T. litoreum*, *T. lutimaris*, *T. mesophilum*, *T. ovolyticum*, *T. sediminilitoris*, *T. skagerrakense*, *T. soleae*, *T. todarotis* and *T. xiamenese*. In addition, we have *T. finnmarkense*, *T. holothuriorum* and *T. japonica* for which the names are not validly published yet.

Tenacibaculosis in fish was first described in 1977 (Masumura & Wakabayashi, 1977). Fish pathogenic species such as *T. maritimum*, *T. dicentrarchi* and *T. finnmarkense* are often associated with ulcers related to the body and mouth, frayed fins, tail rot and necrosis (Avendano-Herrera et al., 2016; Avendano-Herrera, Toranzo, & Magarinos, 2006; Smage et al., 2016).

Tenacibaculum spp. have been isolated from Norwegian salmon and trout aquaculture since the late 1980s (Olsen et al., 2011). In a survey of fish with winter ulcer in 1995-96, *Tenacibaculum* bacteria were found in all 18 sites investigated and in 70% of the ulcers (Takle et al., 2015). In 2004-05, bacteria in genus *Tenacibaculum* were isolated from 62.5% of the ulcers from two sites with winter ulcer disease (Olsen et al., 2011). In Norway, infections with *Tenacibaculum* spp. in Atlantic salmon and rainbow trout (*Oncorhynchus mykiss*) have historically not been systemic and *Tenacibaculum* spp. have rarely been isolated from the head kidney. It is also uncertain whether the *Tenacibaculum* spp. that were isolated from the ulcers have been the primary causative pathogen. It could be that most of the previous isolations of *Tenacibaculum* spp. from ulcers in Norwegian salmon are secondary to another causative agent (Olsen et al., 2011).

T. dicentrarchi was first isolated and described from skin lesions in European sea bass (*Dicentrarchus labrax*) in Spain (Pineiro-Vidal, Gijon, Zarza, & Santos, 2012). In Norway, *T. dicentrarchi* was isolated from Atlantic cod (*Gadus morhua*) in 2009-10 (Habib et.al, 2014). Later investigations resulted in isolation of 19 strains of *Tenacibaculum* spp. in Norway from wrasse (*Labridae*), cod, lumpsucker (*Cyclopterus lumpus*) and Atlantic salmon that were closely related and formed a cluster with *T. dicentrarchi* (Olsen et al., 2017). Those isolations were performed mainly from wrasse. Three of the four salmon isolates they found were from asymptomatic Atlantic salmon. The authors indicate that *T. dicentrarchi* may be more pathogenic to non-salmonid fish than to salmonid fish. In addition, the authors pointed out that isolates from western Norway tend to dominate the *T. dicentrarchi* clade of Norwegian *Tenacibaculum* bacteria.

In Norway, there has been no detailed description of a disease outbreak caused by *T. dicentrarchi* in Atlantic salmon thus far. However, in Chile *T. dicentrarchi* has been associated with tail rot, frayed fins and wounds in severe outbreaks in Atlantic salmon at one site in 2010 and 2014 (Avendano-Herrera et al., 2016).

The aims of this study were to investigate an acute outbreak of disease in an Atlantic salmon farm presumptively caused by *Tenacibaculum* bacteria, identify and genetically characterize the causative agent using multilocus sequence analysis (MLSA) and perform a challenge trial in an Atlantic salmon infection model.

2 | MATERIALS AND METHODS

2.1 | Study population and their management

The land-based post-smolt facility in north-west Norway (Nordmøre) consists of eight circular (diameter 16 m) tanks with a volume of 835 m^3 filled with 750 m^3 sea water when fish are reared in the tanks. The water retention time was 60–70 min. The water came from two different water pipes. The main pipe supplied sea water that was pumped from 80 m depth and heated at 8-10°C above seawater temperature by a heat exchanger before being mixed, to achieve the required temperature of 12°C, with sea water from the second pipe that was supplied by water pumped from a depth of 54 m. After mixing, the water passed through a drum filter with a pore size of 100 μm, before UV disinfection (Wedeco BX 1800, 254 nm wavelength, dose >45 mJ/cm^2), and finally pumped to the tanks. The oxygen content of the water was maintained at 95%–100% saturation in the tanks, and always above 85% at the outflow.

The production cycle in the facility was based on receiving newly smoltified Atlantic salmon (60–100g), rearing them for 3-4 months, before shipping the post-smolts to open net pens at sea sites with a weight of 400–900g. Normally, the facility houses three groups of post-smolt annually.

The smolts were shipped 25 km on public roads by tank trucks in freshwater (approximately 25 000 smolts in each). The smolts were reared in three different tanks in a smolt production facility with recirculated water from a biofilter/bioreactor (RAS). The smolts in the water were transported from the rearing tanks by the trucks to the post-smolt facility. The smolt group shipped comprised a total of 611,429 smolts with an average weight of 78 g. The smolts originated from the same batch of eggs and were grown at the same temperature in the same recirculated freshwater. However, the size of the smolts varied between the three rearing tanks at the smolt facility (74, 75 and 84 g on average). The smolts were unloaded within the shipping water directly from the trucks into eight different post-smolt tanks through pipes (d160mm) by gravity. The post-smolt tanks were prefilled with sea water before receiving the smolts. Each post-smolt tank was loaded with smolts from three trucks before the next tank was loaded. During nine hours the first day (1 November 2016), two tanks (E1 and E2) were filled with smolts and the third (E3) was 2/3 filled with smolts. The next day, the third tank was filled, the fourth (E4) and fifth (F1) tanks were filled, and the sixth (F2) tank was 1/3 filled. On the third and last day of shipment, the sixth, seventh (F3) and eighth (F4) tanks were filled successively. The numbers of smolts in each tank varied from 73,433 (F4) to 79,022 (F1). The smolts were vaccinated with a commercial vaccine (ALPHAJECT micro 6) against *Aeromonas salmonicida* ssp *salmonicida*, *Vibrio (Listonella) anguillarum*, *Aliivibrio salmonicida*, *Moritella viscosa* and infectious pancreatic necrosis (IPN) virus. The salmon had not been exposed to sea water before transfer to the post-smolt facility. The affected post-smolt plant had previously over a few years been experiencing mortality due

to ulcers. Different pathogenic bacteria had frequently been cultivated during the last three years from ulcers and head kidney. *Moritella viscosa*, *Aliivibrio wodanis*, *Vibrio splendidus* and another *Aliivibrio*-like bacterium have been closely associated with the ulcers, and during the last two years.

The farm management had references from other farmers and own experience in application of the water-soluble disinfectant tosylchloramide sodium (chloramine-T) to the rearing water when ulcer disease occurred. After observations of increasing numbers of smolts with ulcers and increased mortality in the rearing tanks, it was decided to treat the water with chloramine-T, weekly from 11 November 2016 at a dose of 10–20 ppm.

2.2 | Clinical and pathological signs

The smolts were visually inspected from walking bridges on top of the tanks during daytime. Dead smolts were collected and counted once daily. All dead smolts were visually inspected daily, and ulcer-affected fish were recorded.

2.3 | Histology

For light microscopy, slices of tissue samples from ulcers, affected fins and liver from two smolts with representative changes were collected and immediately fixed by immersion in 10% buffered formalin and embedded in paraffin. Then, 2- to 3-µm-thick sections were stained with haematoxylin and eosin (HE). The sections were examined under a Nikon Eclipse Ci light microscope, and photomicrographs were captured by a Nikon Infinity 2 camera (Nikon, Tokyo, Japan).

2.4 | Culturing

Totally, 24 moribund fish were sampled (11 fish on 15 November, 11 fish on 23 November and two fish on 16 December). Specimens from the ulcers, fins, gills and kidneys of moribund fish with large lesions were sampled with an inoculation loop and cultured directly on Marine Agar (MA) (Difco™ Marine Broth 2216) and Blood Agar (BA) (OXOID CM0271, Blood Agar Base No. 2, with 2,5% NaCl) and incubated at 12°C for 48–96 hr.

2.5 | Molecular identification and characterization

2.5.1 | Extraction of genomic DNA

Two colonies, convex, circular and pale yellow, from the primary culturing on MA, from each of the different fish were picked and subcultured on MA for 72 hr at 12°C, after which nucleotides were extracted using DNA Purification Kit (Wizard® Genomic DNA Purification Kit (Promega)) as described by the producer.

2.5.2 | Identification using 16S *rRNA* gene analysis

Universal primers (27F and 1492R) and GoTaq® DNA Polymerase Kit (Promega) were used to amplify the 16S *rRNA* gene following the protocol described by the manufacturer. The PCR products were checked for the expected product size, about 1,475 base pairs after running on agarose gel electrophoresis. The purified PCR products were sequenced using sequencing service (GATC, now Eurofins Scientific, Konstanz, Germany).

Identification of the bacteria was done by blasting the 16S *rRNA* gene sequences using Basic Local Alignment Search Tool (BLAST®) at the NCBI website, which compared the nucleotide sequences with the sequence database.

2.5.3 | Multilocus sequence analysis (MLSA)

Based on the results of 16S *rRNA* sequence analysis, we performed multilocus sequence analysis (MLSA) for *Tenacibaculum* spp. using degenerate primers targeting seven housekeeping genes employing the PCR protocol described previously (Habib et al., 2014).

TABLE 1 Source of isolates included in MLSA

Isolate	Date of sampling	Tank	Fish	Organ	Frozen (Luria broth and glycerol)
1	15.11.2016	E4	1	Ulcer	6.12.2016
2	15.11.2016	E4	3	Ulcer	6.12.2016
3	15.11.2016	E4	6	Ulcer	6.12.2016
4	23.11.2016	F1	2	Ulcer	23.12.2016
5	23.11.2016	F1	3	Ulcer	23.12.2016
6	23.11.2016	F1	6	Ulcer	23.12.2016
7	23.11.2016	F1	11	Ulcer	23.12.2016
8	23.11.2016	F1	2	Head Kidney	23.12.2016
9	23.11.2016	F1	6	Head Kidney	23.12.2016
10	23.11.2016	F1	2	Fin lesion	23.12.2016
11	23.11.2016	F1	3	Fin lesion	23.12.2016
12	23.11.2016	F1	6	Fin lesion	23.12.2016

A total of 12 isolates from ulcers, fin lesions and kidney from eight different fish (Table 1) were included in the MLSA. Three of the samples were from those smolts collected on the 15 November (Tank E4), and nine of the samples were from those smolts collected on the 23 November (Tank F1).

The PCR products from each of the genes were purified from single bands and sequenced. Purified water was used as negative control, one in each analysis.

The evolutionary relationship among the current isolates and comparison of isolates from other sources were determined from the phylogenetic trees constructed using the neighbour-joining method (Saitou & Nei, 1987). Phylogenetic trees were constructed using sequences of each of the individual genes as well as concatenated sequences of all the genes. Concatenation of the gene sequences was done in a head-to-tail manner according to their physical order in the sequenced genome of the *Tenacibaculum* strain (Accession No. AY7486TD) (Grothusen et al., 2016). The evolutionary distance was computed using the maximum composite likelihood method (Tamura, Nei, & Kumar, 2004), and the evolutionary analyses were conducted in MEGA7 (Kumar, Stecher, & Tamura, 2016).

2.5.4 | Challenge trials

First trial

Atlantic salmon used in this trial were unvaccinated from Sørsmolt AS, Sannidal, Kragerø, and were about 40 g at the start of the trial. The fish were kept in freshwater for 15 days before the challenge and then gradually transferred to sea water and kept in full sea water, salinity 34.6 mg/g, for 11 days before challenge. The water temperature was 8 ^{O}C. All 227 salmon were pit (passive integrated transponders)-tagged intraperitoneally and were kept in the same 1,400-litre tank.

The challenge strain was prepared from a frozen stock culture of *T. dicentrarchi*, isolate 8 in the MLSA study. The *T. dicentrarchi* isolate selected for challenge was cultivated from a single colony on Marine Agar and subcultured on Marine Broth for 24 hr at 10°C with shaking (130 rpm). The start volume was 5 ml with increasing amount of Marine Broth up to 4,000 ml over nine days before using the culture for the challenge trial at a bacterial concentration of 3.6×10^8 cfu/ml.

In total, 30 shedders were anaesthetized with benzocaine (30 mg/L) and then challenged with 0.1 ml of *T. dicentrarchi* suspension (3.6×10^7 cfu) ip each. The shedders were then put back into the 1,400-litre tank. The water level was lowered to 400 l, the water supply stopped, and four litres of *T. dicentrarchi* suspension was added (bacterial concentration of the water with fish was 3.6×10^6 cfu/ml). After 30 min, the water supply was started. During the challenge, the salmon got extra oxygen, by rubber tube dispensers with multiple small holes.

The salmon were followed for 98 days after challenge during which mortality and ulcers were registered. At the end of the study, all the fish were euthanized, weighed and checked for ulcers.

Second trial

The 36 Atlantic salmon used in this trial were unvaccinated from NMBUs own hatchery and smolt facility at Ås, Akershus, and were on average 57 g at challenge. The fish were stocked in a freshwater 1,400-litre tank. Five days before the challenge, the fish were moved to a 180-litre tank and then gradually transferred to sea water, and kept in full sea water, 34.6 mg/g, the day before challenge. The water temperature was 8.9°C. The salmon appeared to be optimally smoltified. All 36 smolts were pit-tagged and were kept in the same 180-litre tank. A total of 26 smolts were anaesthetized with benzocaine (30 mg/L) and scarified (1 cm × 1 cm) by removing scales approximately 1 cm below the dorsal fin. The scales were removed by scraping with a scalpel. For 20 of the scarified fish, we applied three drops of the bacterial culture directly on the scarified area for 15 s. Twenty minutes after the scarification of the last fish, all 36 fish were bath-challenged for two hours (4×10^7 cfu/ml). Before challenge, the water level in the tank was decreased to 160 litres and 2 l of *T. dicentrarchi* suspension was added (bacterial concentration: 3×10^9 cfu/ml). During the challenge, the salmon got extra oxygen and the O_2 during challenge was 8.3 mg/L. After 2 hr, the water supply was started and the water level was raised to 180 litres. Water retention time in the tank was 30 min. The salmon were observed until all the salmon were dead or moribund. Mortality and ulcers were registered, and all fish were necropsied. The challenge strain was the same as in the first trial and was prepared from frozen stock to challenge the same way as in the first trial.

Specimens from the skin with artificial scale loss, fins, liver and kidneys of dead and moribund fish were sampled with an inoculation loop and cultured directly on Marine Agar (MA) (Difco™ Marine Broth 2216) and Blood Agar (BA) (OXOID CM0271, Blood Agar Base No. 2, with 2,5% NaCl) and incubated at 10°C for 3-5 days. We also cultivated blood samples from two moribund salmon, spread 0.1 ml with an L-shaped spreader on both MA and BA and incubated at 10°C for 3–5 days.

For control, ten pit-tagged fish from the same group and the same 1,400-litre freshwater tank were placed in a similar tank of 180 litre volume, with similar water supply as the challenged fish.

The challenge study was approved by the Norwegian Food and Safety Authority, FOTS ID 2017/12388.

3 | RESULTS

3.1 | Mortality and pathological signs

An increased mortality was observed in Tank E1, the tank into which the smolts were transferred first from the shipping trucks, seven days after the smolt arrived at the post-smolt facility. In parallel smolts with ulcers caudal to the pectoral fin were observed in the same tank. After three more days, the mortality rate was increasing in all the tanks (Figure 1 and table in Appendix 1). The mortality declined and was close to normal, less than 0.01%, two to three weeks after the onset and rise in mortality.

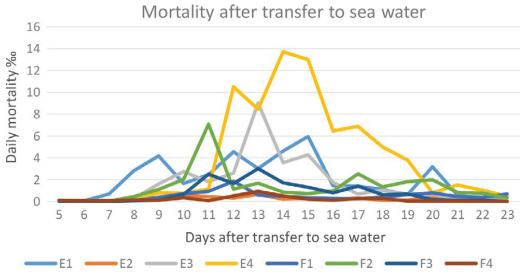

FIGURE 1 Daily mortality of smolts in each tank the first month after transfer. The mortality increased 6–9 days after transfer to sea water, peaking 10–16 days before the mortality declined

The total mortality in the four weeks from arrival of the smolt at the post-smolt site to the end of the outbreak in the different tanks varied from 0.5% (Tank F4) to 7.6% (Tank E4). The highest daily mortality in a single tank was 1.4% (Tank E4).

The moribund and dead salmon had severe lesions, often 2 to 6 cm in width, particularly behind the pectoral fins (Figures 2 and 3). The lesions penetrated the skin and often extended into the muscle tissue. In some of the moribund salmon, the lesions perforated the abdominal wall resulting in exposure of the internal organs (gut and liver) to the sea water. Fin rot particularly on the pectoral fins was also observed.

3.2 | Histology and microscopy

In sections from ulcers and fins, an overwhelming appearance of long, slender *Tenacibaculum*-like bacteria was observed. The bacteria were seen in dermis and also penetrating deep into intramuscular connective tissues and in muscle fibres nearby ulcers. The tissues were highly degenerated and were observed as dissolved nearby the ulcers (Figures 4 and 5).

No bacteria were observed in histological sections from liver of affected smolts.

Microscopic examination of smears from ulcers and head kidney revealed overwhelming appearance of long, slender *Tenacibaculum*-like bacteria.

3.3 | Bacteria isolation and identification

Cultivation from fish sampled on both the 15 and the 23 November revealed similar growth of bacteria. Culturing from ulcers and gills on blood agar resulted in sparse growth of a mixed flora of three to four different bacteria. Cultivation from wounds on Marine Agar resulted in a very rich and pure growth of pale yellow, circular and convex colonies (Figure 6). Cultivation from the head kidney on Marine Agar resulted in similar colonies as were seen from ulcers, but in fewer numbers. Gram staining and microscopic examination of these pale yellow colonies revealed the same as seen from the lesions or ulcers, that is long and thin Gram-negative rod-shaped *Tenacibaculum*-like bacteria.

Cultivation from fish sampled on the 16 December, that is between two and three weeks after the end of the outbreak, revealed growth of a mixed flora from ulcers. No growth of *Flavobacterium* or *Tenacibaculum*-like bacteria was observed on Marine Agar. At this time, there were few moribund fish to catch, and only two fish were sampled for bacteriological analysis.

3.4 | 16S rRNA analysis

The results from the BLAST® using 16S rDNA sequence from the two bacterial colonies that were analysed were similar to, but gave no complete match to a particular bacterial species, suggesting this bacterium may have not been described earlier. However, it was indicative that the bacteria were a *Tenacibaculum* species. The closest bacteria from the BLAST® result were *T. dicentrarchi.* The *T. dicentrarchi*-type strain (35/09) and the query strain differed in six of the 830 base pairs between base pair nr 70 and 900 for the type strain (data not shown).

3.5 | Phylogenetic analysis based on housekeeping genes

Phylogenetic analysis based on the individual gene trees showed consistent results where all the *Tenacibaculum* isolates obtained from the current study were generally clustered with *T. dicentrarchi* strains from Chile (TdChD01, 02, 03, 04, 05 and 06) and Spain (USC35/09, NCIMB 14598) supported by high bootstrap values

FIGURE 2 Moribund smolts from Tank E4 euthanized on the 15 November 2016 with typical ulcers caudal to the breast fin on the left side of the fish

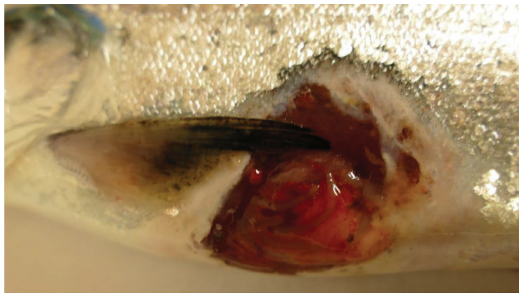

FIGURE 3 Photograph of a moribund smolt with an open ulcer from Tank E4 euthanized on the 15 November 2016

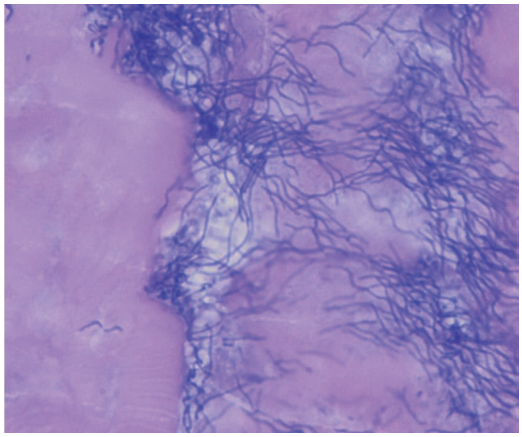

FIGURE 4 Histological section from ulcers revealed many thread-formed *T. dicentrarchi* cells penetrating deep into the musculature surrounding ulcers(x1000)

(83%–99%) (Figure 7). The *Tenacibaculum* isolates obtained from the current study also clustered with a Norwegian isolate from cod (TNO012), which was clustered with *T. dicentrarchi* (Olsen et al., 2017). The 12 isolates from the current study seem to be a clonal complex or similar genotype since all were tightly clustered except isolate 05, which was clustered a bit separately in most gene trees though all were located within the *T. dicentrarchi* cluster. Of all the genes, the *gyrB* gene showed the highest resolution in which case the current isolates were located in two genotypes, one genotype comprising 10 isolates clustered with *T. dicentrarchi* from Chile and the other comprising two isolates (isolates 2 and 10) clustered with *T. dicentrarchi* from Spain and other strains from Chile (Figure in Appendix 1).

3.6 | Challenge trial

3.6.1 | First trial

After challenge, the fish neither developed ulcers nor died.

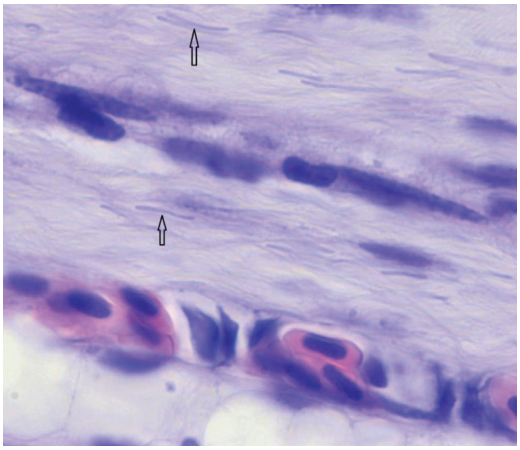

FIGURE 5 Histological section from an affected tail fin revealed many long, slender *Tenacibaculum*-like bacteria (arrows) penetrating deep into the tissue nearby affected fins (x1000)

3.6.2 | Second trial

After 24 hr from the challenge with *T. dicentrarchi*, 32 post-smolts were dead and two post-smolts that were moribund were killed. Then, in addition after 48 hr from the challenge the two remaining post-smolts were dead, too. Of the four fish still living after 24 hr, one was scarified without applying drops of bacteria in the scarified area and three were not scarified.

All the fish had extensive scale loss, small haemorrhagic lesions particularly close to the lateral line, ascites and dark liver (Figure 8). Some of the fish had also corneal ulcers and haemorrhage in the eye. The four fish that were alive after 24 hr had in addition small ulcers and rubor in the head and jaws (Figure 9).

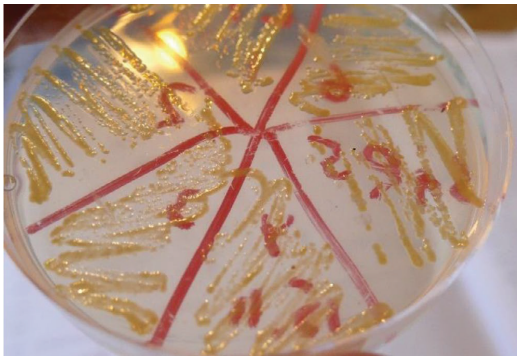

FIGURE 6 Cultivation on Marine Agar from ulcers revealed abundant growth of pale yellow, circular and convex colonies

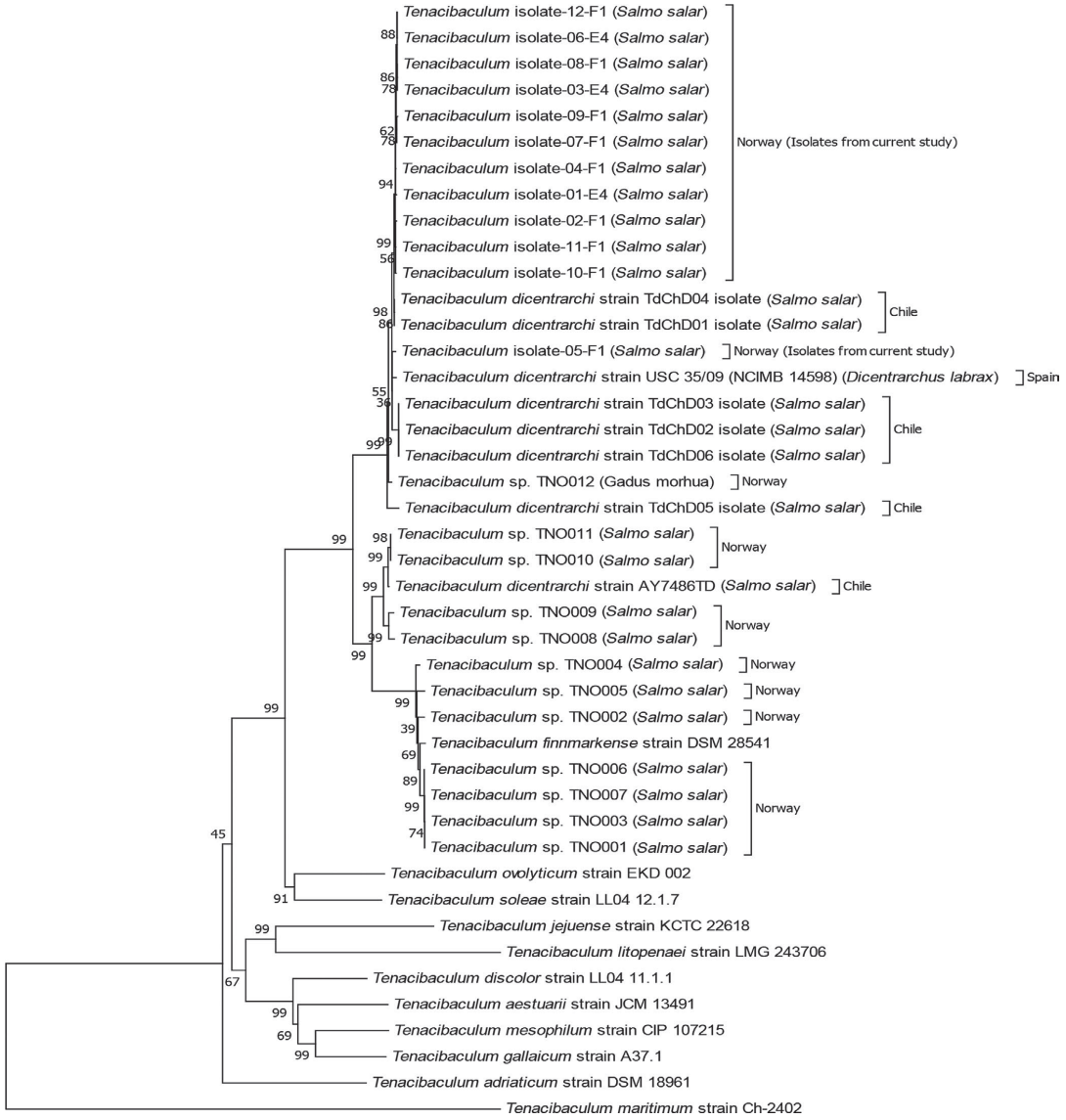

FIGURE 7 Evolutionary relationships of taxa based on concatenated partial gene sequences of the described seven housekeeping genes. The evolutionary history was inferred using the neighbour-joining method. The optimal tree with the sum of branch length = 1.48073827 is shown. The confidence probability (multiplied by 100) that the interior branch length is greater than 0 as estimated using the bootstrap test (1,000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the maximum composite likelihood method and are in the units of the number of base substitutions per site. The differences in the composition bias among sequences were considered in evolutionary comparisons. The analysis involved 43 nucleotide sequences. Codon positions included were 1st+2nd+3rd+non-coding. All positions containing gaps and missing data were eliminated. There were a total of 3,662 positions in the final data set. Evolutionary analyses were conducted in MEGA7

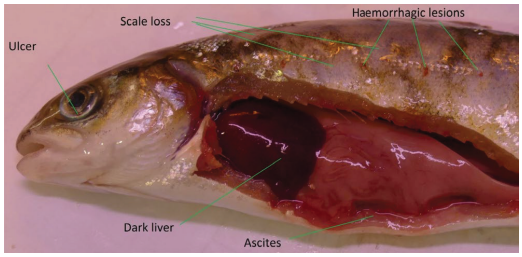

FIGURE 8 Dead fish 24 hr after challenge with *T. dicentrarchi*

FIGURE 9 Moribund fish 24 hr after challenge with *T. dicentrarchi*. In addition to the dark liver, ascites, corneal ulcers, scale loss and haemorrhagic lesions that were observed in the dead fish, the moribund fish also had rubor and lesions in head and jaws

3.6.3 | Cultivation from the fish

Cultivation was negative on blood agar after incubation of material from liver, head kidney and blood. From wounds and scale loss, there was sparse growth of mixed flora. Cultivation from lesions and scale loss on Marine Agar resulted in rich and almost pure growth of pale yellow mostly circular convex colonies similar to the isolate of the *T. dicentrarchi* employed in the challenge. From head kidneys, there was growth of similar colonies from 17 of the 36 fish and from five of the 17 livers we cultivated from. Cultivation from blood of the two moribund fish we killed 24 hr after the challenge results in growth of similar *T. dicentrarchi* colonies. From blood, we cultivated 330 cfu/ml from one fish and more than 1,000 cfu/ml from the other fish. Microscopic examination of the smears from lesions and from the colonies from blood, head kidney, lesions and liver revealed long and thin rod-shaped *Tenacibaculum*-like bacteria.

No mortality or pathological signs were recorded in the control group.

4 | DISCUSSION

Isolation of *T. dicentrarchi* mainly in monoculture from the wounds of diseased fish as well as from the head kidney and the observation of massive invasion of thread-formed Gram-negative bacteria, resembling *T. dicentrarchi*, from histological sections of the wounds and infected fins suggest that *T. dicentrarchi* was the causative agent of disease and mortality in the current outbreak. This conclusion is supported by 100% mortality within 48 hr after challenge with *T. dicentrarchi* in the second challenge trial and growth of *Tenacibaculum*-like bacteria from scale loss, kidneys and blood from specimen in that trial.

Tenacibaculosis has probably been a major problem in north-west Norway in the spring of 2018 for smolts after transfer to sea. Fish farmers in the area communicate that mortality is probably caused by acute tenacibaculosis that starts 1–3 weeks after transfer of Atlantic salmon smolts from freshwater in the smolt plants to the sea sites and with a decline 1–3 weeks later. (Tidens Krav, 2018). This development of mortality and probable successive autovaccination is similar to what was registered at the post-smolt site in the outbreak described in this study. *T. dicentrarchi* could have been the causative agent of some of the tenacibaculosis outbreaks seen in north-west Norway in the spring of 2018.

Water treatment with chloramine-T at the post-smolt facility may have contributed to the rapid decline in mortality. However, the mortality in the tanks was at the highest five to six days after the first chloramine-T treatment, which indicates that the chloramine-T treatment did not eliminate the pathogen. Antiseptics such as chloramine-T act topically and will probably not affect the *T. dicentrarchi* cells already established deep into the tissues of the fish, and will therefore have minor effects on already well-established bacterial infections in the tissues.

Since *Tenacibaculum* spp. are only isolated from the marine environment and since they require NaCl for growth in artificial media (Suzuki et al., 2001), it is unlikely that the fish have been infected with *T. dicentrarchi* at the freshwater site. The observation that the first mortality occurred within a week after transfer indicates that the fish were most probably infected immediately after being transferred to the post-smolt site.

The period from seawater transfer to the first mortality from infection by *T. dicentrarchi* at the post-smolt facility was six to nine days at 12°C degrees. After the onset of mortality, the mortality rose for two to four days, followed by a decrease, and again rose for five to seven days after the first onset of mortality. The second increase in mortality may be due to a new rise in the concentration of infective bacterial cells in the water shedded from the first group of diseased salmon. The shorter interval between the first and second increase in mortality compared to the interval between transfer to sea water and the first increase in mortality is probably caused by shedding of bacteria started one to two days before the onset of the first mortality, and four to six days after the first contact with the water with *T. dicentrarchi*. The density of *T. dicentrarchi* might also be higher in the shedding period than in the first period in sea water.

The analysis of nucleotide sequences from the seven housekeeping genes demonstrated that none of the 12 isolates of *T. dicentrarchi*

had identical DNA sequences in all seven genes. Eleven of 12 isolates from the studied tenacibaculosis outbreak have relatively similar genotypes as seen from the clustering in the phylogenetic trees generated from the MLSA (Figure 7). The 12th isolate (05-F1) had a genotype that diverges from the other 11 isolates. This variation in DNA for all the 12 isolates indicates a co-infection with several genotypes of *T. dicentrarchi*. The finding that the DNA sequences of all the studied isolates have minor variations despite the fact that the isolates were obtained from the same site and the same outbreak of disease, and some also from the same tank and the same fish, partly supports previous reports that *Tenacibaculum* infections often involve multiple strains that lack strain clonality even from the same disease cases, and change genotype rapidly (Olsen et al., 2017). This type of co-infection is common in bacterial diseases in fish and has also been reported for many other *Flavobacterium* sp. (Fujiwara-Nagata et al., 2013; Kinnula, Mappes, & Sundberg, 2017). Higher virulence has also been reported in co-infections than in single-genotype infections (Kinnula et al., 2017; Louhi, Sundberg, Jokela, & Karvonen, 2015), which may highlight the role of interaction between genotypes within the same species in developing pathogenicity. When the methods for differentiating genotypes become more accurate, it will not be surprising to find many more different genotypes involved in a single case opposite to what we previously thought was caused by a single genotype. Full-genome sequencing may reveal such detailed variation. In fact, this study may not demonstrate good resolution of all the genotypes of the studied isolates since we employed housekeeping genes in MLSA, which are in most cases highly conserved. The phylogenetic distance between the studied isolates could have been larger if genes showing short evolutionary history, such as virulence genes, were used in the analysis.

The documentation of occurrence of different genotypes of *T. dicentrarchi* in this outbreak indicates that the infectious agent arrives with the water.

The involvement of genetically diverse isolates associated with an outbreak of tenacibaculosis has made it difficult to select representative candidate strains for vaccine development (Olsen et al., 2017). Previous reports conclude that most of the *Tenacibaculum* species isolated from Norway, except *T. maritimum*, share a common ancestry (Olsen et al., 2017). Because of this common ancestry, there is an expectation that *Tenacibaculum* spp could possibly share some characteristics including virulence or antigenic determinants. This will probably make it easier to develop a broader vaccine against different *Tenacibaculum* strains. The observation of a rapid decline in mortality after approximately three weeks in the natural outbreak may suggest a quick and adequate immune response in clearing the infection. This might also indicate a potential good window of opportunity to make an effective vaccine since the time from exposure to protection was short due to possible autovaccination.

We did not get any mortality or development of ulcer in our first challenge trial. The route of administration, particularly the way the bath challenge was carried out without scarification of the skin and the short time of exposure to the pathogen, may be factors that limited pathogenicity during the challenge experiment. Empirically, it has been difficult to induce tenacibaculosis caused by *Tenacibaculum (*Masumura & Wakabayashi, 1977*)* (Lunder, Evensen, Holstad, & Håstein, 1995; Olsen et al., 2011; Powell, Carson, & van Gelderen, 2004). Often a mechanical skin damage has been necessary to cause ulcer or disease (Baxa, Kawai, & Kusuda, 1987; Olsen et al., 2011; Wakabayashi, Hikida, & Masumura, 1984).

It has also been indicated that an exposure of one to two hours of *T. maritimum* is not sufficient to develop a biofilm on the skin that is required for a successful infection of turbot (Avendano-Herrera et al., 2006). In a successful experimental induction of mouth rot in Atlantic salmon smolt using *T. maritimum*, both the concentration of bacterial cells/ml and the challenge time were higher than used in this study (Frisch et al., 2018).

The temperature of 8°C used in the challenge trial was four degrees lower than the water temperature in the outbreak at the post-smolt site. The bacteria may need more time at 8°C to develop a biofilm, or attach and colonize sufficiently and do harm to the fish than for instance at 12°C.

In our second challenge trial, we got 89% mortality within 24 hr and 100% mortality within 48 hr. The isolate of *T. dicentrarchi* employed was the same as in the first trial. The concentration of bacteria in the water was higher, and the challenge time was longer in the second trial, 4×10^7 cfu/ml vs. 3.6×10^6 cfu/ml and 120 min vs. 30 min. Most of the fish in the second trial were also scarified, but there were also mortality and disease in non-scarified fish. There were, however, more rapid onset of mortality in the scarified fish where 96% died within 24 hr vs. 70% in the non-scarified fish.

The bath challenge of Atlantic salmon with *T. dicentrarchi* in Chile (Avendano-Herrera et al., 2016) in which 65% of the Atlantic salmon died was performed in water at 16°C, without scarification using a concentration of 3.78×10^5 cfu/ml and a challenge time for 60 min. The Chilean trial was performed with bacteria from one single strain of *T. dicentrarchi* (TdCdD04) (Avendano-Herrera et al., 2016). This strain is genetically very close to the strains we found in the described outbreak (Figure 7).

It is difficult to explain why disease developed in one trial and not in another. The variation could be due to a combination of one or more factors including a higher pathogenic capacity of the Chilean strain, the cumulative mortality in the natural outbreaks in Chile reached 60% (Avendano-Herrera et al., 2016), more susceptible Chilean salmon, a higher challenge dose in the Chilean trial or a higher water temperature in Chile. One of the reasons for the more rapid onset of mortality in our second challenge trial despite colder water compared to the natural outbreak was probably the high challenge dose.

The immune system is changed after stress, and the susceptibility of infectious diseases may increase after stress (Fast, Hosoya, Johnson, & Afonso, 2008). The salmon in the post-smolt facility were probably stressed when transferred to the site, after crowding, pumping from the fresh water site to the trucks, transported on bumpy roads and pumped again into the tanks at the post-smolt site (Lerfall et al., 2015). This stress has probably made the salmon extra susceptible to infection with *T. dicentrarchi*. The mechanical handling during transfer could also injure some of the salmon, for

instance by creation of lesions in the epidermis and dermis. If lesions in the dermis or epidermis occurred during transfer, then the salmon are probably more susceptible to the infection, since scarification is often necessary to cause tenacibaculosis (Baxa et al., 1987; Olsen et al., 2011; Wakabayashi et al., 1984).

Tenacibaculosis is one of the diseases that often has been difficult to reproduce, and therefore, it could be challenging to fulfil Koch's postulates with *Tenacibaculum sp*. Koch's postulates are useful for some diseases, but are of limited usefulness for other diseases, particularly multifactorial ones (Ross & Woodward, 2016). For multifactorial diseases, it is often not enough to introduce the contagious agent to cause disease. Other parameters, for instance immune competence, age of animal, route of application and genetic profile for both microbe and the host, will also influence experimental host–pathogen interactions and co-determine whether disease occurs or not after challenge (Hess, 2017). Commensal bacteria can also protect the host against pathogenic bacteria in some cases, and microbial interactions and microbial communities should therefore also be incorporated into disease causation (Byrd & Segre, 2016).

The occurrence of acute tenacibaculosis at the post-smolt site in this study is alarming due to the fact that the intake water is filtered through a 100-µm filter before the water is UV-treated with a UV dose of at least 45 mJ/cm^2, well above the national requirements in Norway which is 25 mJ/cm^2. This indicates that UV disinfection at doses of 45 mJ/cm^2 may not be sufficient to prevent bacteria such as *Tenacibaculum* and probably other bacterial pathogens of fish from causing disease at post-smolt sites. The occurrence and outbreaks of other bacterial diseases caused by *Moritella viscosa*, *Vibrio splendidus* and *Aliivibrio wodanis* at the same site further corroborate the ineffectiveness of UV disinfection. This warrants reconsideration or further research on the relevance of UV disinfection. It has been shown that different bacterial groups have different levels of susceptibility to UV disinfection and that at least some marine bacteria show tendency to recover after disinfection treatment (Moreno-Andres, Acevedo-Merino, & Nebot, 2018). In addition, UV radiation penetrates only superficially in the matrix of biofilms and often only the few top layers of bacterial cells will be killed by UV disinfection if the bacteria are protected in biofilms (de Carvalho, 2017).

In conclusion, an acute outbreak of tenacibaculosis in Atlantic salmon at a post-smolt site in north-western Norway was described and its causative agent was characterized. Genetic characterization of 12 isolates obtained from diseased post-smolt using multilocus sequence analysis (MLSA) revealed that all isolates clustered with *T. dicentrarchi*.

ACKNOWLEDGEMENTS

The work was supported by the Norwegian Research Council (Grant No. 260204/O30) and Previwo AS.

CONFLICT OF INTERESTS

The authors declare that they have no conflict of interest.

ORCID

Øystein Klakegg https://orcid.org/0000-0001-7024-3612

REFERENCES

Avendano-Herrera, R., Irgang, R., Sandoval, C., Moreno-Lira, P., Houel, A., Duchaud, E., … Ilardi, P. (2016). Isolation, Characterization and Virulence Potential of Tenacibaculum dicentrarchi in Salmonid Cultures in Chile. *Transboundary and Emerging Diseases*, *63*(2), 121–126. https://doi.org/10.1111/tbed.12464

Avendano-Herrera, R., Toranzo, A. E., & Magarinos, B. (2006). Tenacibaculosis infection in marine fish caused by Tenacibaculum maritimum: A review. *Diseases of Aquatic Organisms*, *71*(3), 255–266. https://doi.org/10.3354/dao071255

Baxa, D. V., Kawai, K., & Kusuda, R. (1987). Experimental-infection of flexibacter-maritimus in black-sea bream (Acanthopagrus-Schlegeli) fry. *Fish Pathology*, *22*(2), 105–109. https://doi.org/10.3147/jsfp.22.105

Byrd, A. L., & Segre, J. A. (2016). Infectious disease. Adapting Koch's postulates. *Science*, *351*(6270), 224–226. https://doi.org/10.1126/science.aad6753

de Carvalho, C. (2017). Biofilms: Microbial Strategies for Surviving UV Exposure. *Advances in Experimental Medicine and Biology*, *996*, 233–239. https://doi.org/10.1007/978-3-319-56017-5_19

Fast, M. D., Hosoya, S., Johnson, S. C., & Afonso, L. O. (2008). Cortisol response and immune-related effects of Atlantic salmon (Salmo salar Linnaeus) subjected to short- and long-term stress. *Fish & Shellfish Immunology*, *24*(2), 194–204. https://doi.org/10.1016/j.fsi.2007.10.009

Frisch, K., Småge, S. B., Vallestad, C., Duesund, H., Brevik, O. J., Klevan, A., … Nylund, A. (2018). Experimental induction of mouthrot in Atlantic salmon smolts using Tenacibaculum maritimum from Western Canada. *Journal of Fish Diseases*, https://doi.org/10.1111/jfd.12818

Fujiwara-Nagata, E., Chantry-Darmon, C., Bernardet, J. F., Eguchi, M., Duchaud, E., & Nicolas, P. (2013). Population structure of the fish pathogen Flavobacterium psychrophilum at whole-country and model river levels in Japan. *Veterinary Research*, *44*, 34. https://doi.org/10.1186/1297-9716-44-34

Grothusen, H., Castillo, A., Henriquez, P., Navas, E., Bohle, H., Araya, C., … Mancilla, M. (2016). First complete genome sequence of *Tenacibaculum dicentrarchi*, an emerging bacterial pathogen of salmonids. *Genome Announc*, *4*(1), https://doi.org/10.1128/genomeA.01756-15

Habib, C., Houel, A., Lunazzi, A., Bernardet, J.-F., Olsen, A. B., Nilsen, H., … Duchaud, E. (2014). Multilocus sequence analysis of the marine bacterial genus Tenacibaculum suggests parallel evolution of fish pathogenicity and endemic colonization of aquaculture systems. *Applied and Environment Microbiology*, *80*(17), 5503–5514. https://doi.org/10.1128/aem.01177-14

Hess, M. (2017). Commensal or pathogen - a challenge to fulfil Koch's Postulates. *British Poultry Science*, *58*(1), 1–12. https://doi.org/10.1080/00071668.2016.1245849

Institute of Marine Research (2018). Research.https://www.hi.no/temasider/sykdom_virus_bakterier_parasitter/sykdom_bakterier_virus_og_parasitter/tenacibaculum_spp./nb-no.

Kinnula, H., Mappes, J., & Sundberg, L. R. (2017). Coinfection outcome in an opportunistic pathogen depends on the inter-strain interactions. *BMC Evolutionary Biology*, *17*(1), 77. https://doi.org/10.1186/s12862-017-0922-2

Kumar, S., Stecher, G., & Tamura, K. (2016). MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Molecular Biology and Evolution*, *33*(7), 1870–1874. https://doi.org/10.1093/molbev/msw054

Lerfall, J., Roth, B., Skare, E. F., Henriksen, A., Betten, T., Dziatkowiak-Stefaniak, M. A., & Rotabakk, B. T. (2015). Pre-mortem stress and

the subsequent effect on flesh quality of pre-rigor filleted Atlantic salmon (Salmo salar L.) during ice storage. *Food Chemistry*, *175*, 157–165. https://doi.org/10.1016/j.foodchem.2014.11.111

Louhi, K. R., Sundberg, L. R., Jokela, J., & Karvonen, A. (2015). Interactions among bacterial strains and fluke genotypes shape virulence of co-infection. *Proceedings of the Royal Society B: Biological Sciences*, *282*(1821), 20152097. https://doi.org/10.1098/rspb.2015.2097

Lunder, T., Evensen, Ø., Holstad, G., & Håstein, T. (1995). 'Winter ulcer' in the Atlantic salmon Salmo salar. Pathological and bacteriological investigations and transmission experiments. *Diseases of Aquatic Organisms*, *23*(1), 39–49.

Masumura, K., & Wakabayashi, H. (1977). An Outbreak of gliding bacterial disease in hatchery-born red seabream (Pagrus major) and Gilthead (*Acanthopagrus schlegeli*) fry in Hiroshima. *Fish Pathology*, *12*(3), 171–177. https://doi.org/10.3147/jsfp.12.171.

Moreno-Andres, J., Acevedo-Merino, A., & Nebot, E. (2018). Study of marine bacteria inactivation by photochemical processes: Disinfection kinetics and growth modeling after treatment. *Environmental Science and Pollution Research International*, *25*(28), 27693–27703. https://doi.org/10.1007/s11356-017-1185-6

Olsen, A. B., Gulla, S., Steinum, T., Colquhoun, D. J., Nilsen, H. K., & Duchaud, E. (2017). Multilocus sequence analysis reveals extensive genetic variety within Tenacibaculum spp. associated with ulcers in sea-farmed fish in Norway. *Veterinary Microbiology*, *205*, 39–45. https://doi.org/10.1016/j.vetmic.2017.04.028

Olsen, A. B., Nilsen, H., Sandlund, N., Mikkelsen, H., Sørum, H., & Colquhoun, D. J. (2011). Tenacibaculum sp. associated with winter ulcers in sea-reared Atlantic salmon Salmo salar. *Diseases of Aquatic Organisms*, *94*(3), 189–199. https://doi.org/10.3354/dao02324

Pineiro-Vidal, M., Gijon, D., Zarza, C., & Santos, Y. (2012). Tenacibaculum dicentrarchi sp. nov., a marine bacterium of the family Flavobacteriaceae isolated from European sea bass. *International Journal of Systematic and Evolutionary Microbiology*, *62*(Pt 2), 425–429. https://doi.org/10.1099/ijs.0.025122-0

Powell, M., Carson, J., & van Gelderen, R. (2004). Experimental induction of gill disease in Atlantic salmon Salmo salar smolts with *Tenacibaculum maritimum*. *Diseases of Aquatic Organisms*, *61*(3), 179–185. https://doi.org/10.3354/dao061179

Ross, L. N., & Woodward, J. F. (2016). Koch's postulates: An interventionist perspective. *Studies in History and Philosophy of Biological and Biomedical Sciences*, *59*, 35–46. https://doi.org/10.1016/j.shpsc.2016.06.001

Saitou, N., & Nei, M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, *4*(4), 406–425. https://doi.org/10.1093/oxfordjournals.molbev.a040454

Småge, S. B., Brevik, O. J., Duesund, H., Ottem, K. F., Watanabe, K., & Nylund, A. (2016). Tenacibaculum finnmarkense sp. nov., a fish pathogenic bacterium of the family Flavobacteriaceae isolated from Atlantic salmon. *Antonie Van Leeuwenhoek*, *109*(2), 273–285. https://doi.org/10.1007/s10482-015-0630-0

Suzuki, M., Nakagawa, Y., Harayama, S., & Yamamoto, S. (2001). Phylogenetic analysis and taxonomic study of marine Cytophaga-like bacteria: Proposal for Tenacibaculum gen. nov. with Tenacibaculum maritimum comb. nov. and Tenacibaculum ovolyticum comb. nov., and description of Tenacibaculum mesophilum sp. nov. and Tenacibaculum amylolyticum sp. nov. *International Journal of Systematic and Evolutionary Microbiology*, *51*(Pt 5), 1639–1652. https://doi.org/10.1099/00207713-51-5-1639

Takle, H. R., Ytteborg, E., Nielsen, K. V., Karlsen, C. R., Nilsen, H. K., Sveen, L., Nilsen, A.. (2015). Sårproblematikk og hudhelse i laks-og regnbueørrettoppdrett.

Tamura, K., Nei, M., & Kumar, S. (2004). Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proceedings of the National Academy of Sciences*, *101*(30), 11030–11035. https://doi.org/10.1073/pnas.0404206101

Tidens Krav Myre, A., (2018). https://www.tk.no/nyheter/smola/halsa/minst-500-000-laksesmolt-har-krepert-pa-n-maned-dramatisk-og-uakseptabelt-sier-mattilsynet/s/5-51-468119.

Wakabayashi, H., Hikida, M., & Masumura, K. (1984). Flexibacter infection in cultured marine fish in Japan. *Helgolander Meeresuntersuchungen*, *37*(1–4), 587–593.

How to cite this article: Klakegg Ø, Abayneh T, Fauske AK, Fülberth M, Sørum H. An outbreak of acute disease and mortality in Atlantic salmon (*Salmo salar*) post-smolts in Norway caused by *Tenacibaculum dicentrarchi*. *J Fish Dis*. 2019;42:789–807. https://doi.org/10.1111/jfd.12982

APPENDIX 1

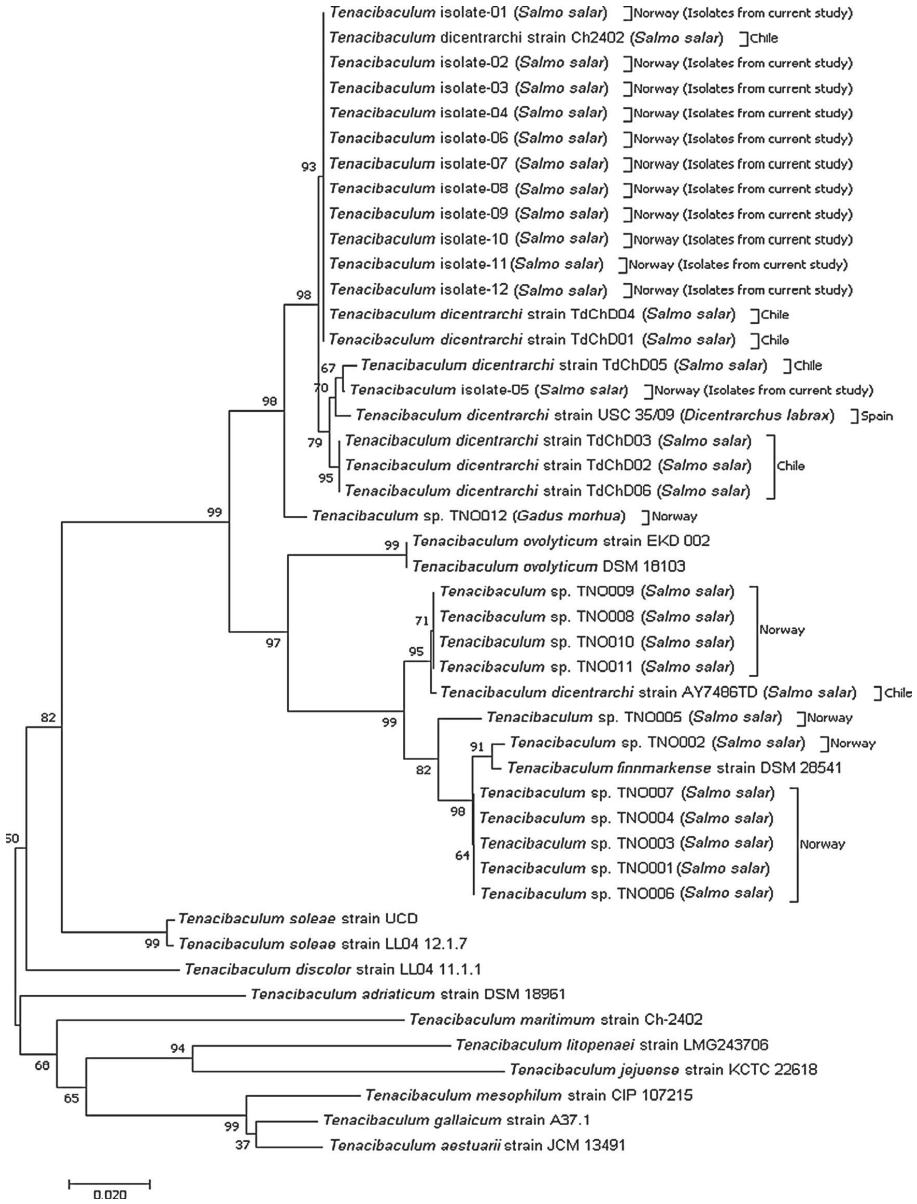

FIGURE A1 Evolutionary relationships of taxa based on *atpA* gene. The evolutionary history was inferred using the neighbour-joining method. The optimal tree with the sum of branch length = 0.70906972 is shown. The confidence probability (multiplied by 100) that the interior branch length is greater than 0 as estimated using the bootstrap test (1,000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the maximum composite likelihood method and are in the units of the number of base substitutions per site. The differences in the composition bias among sequences were considered in evolutionary comparisons. The analysis involved 46 nucleotide sequences. Codon positions included were 1st+2nd+3rd+non-coding. All positions containing gaps and missing data were eliminated. There were a total of 567 positions in the final data set. Evolutionary analyses were conducted in MEGA7

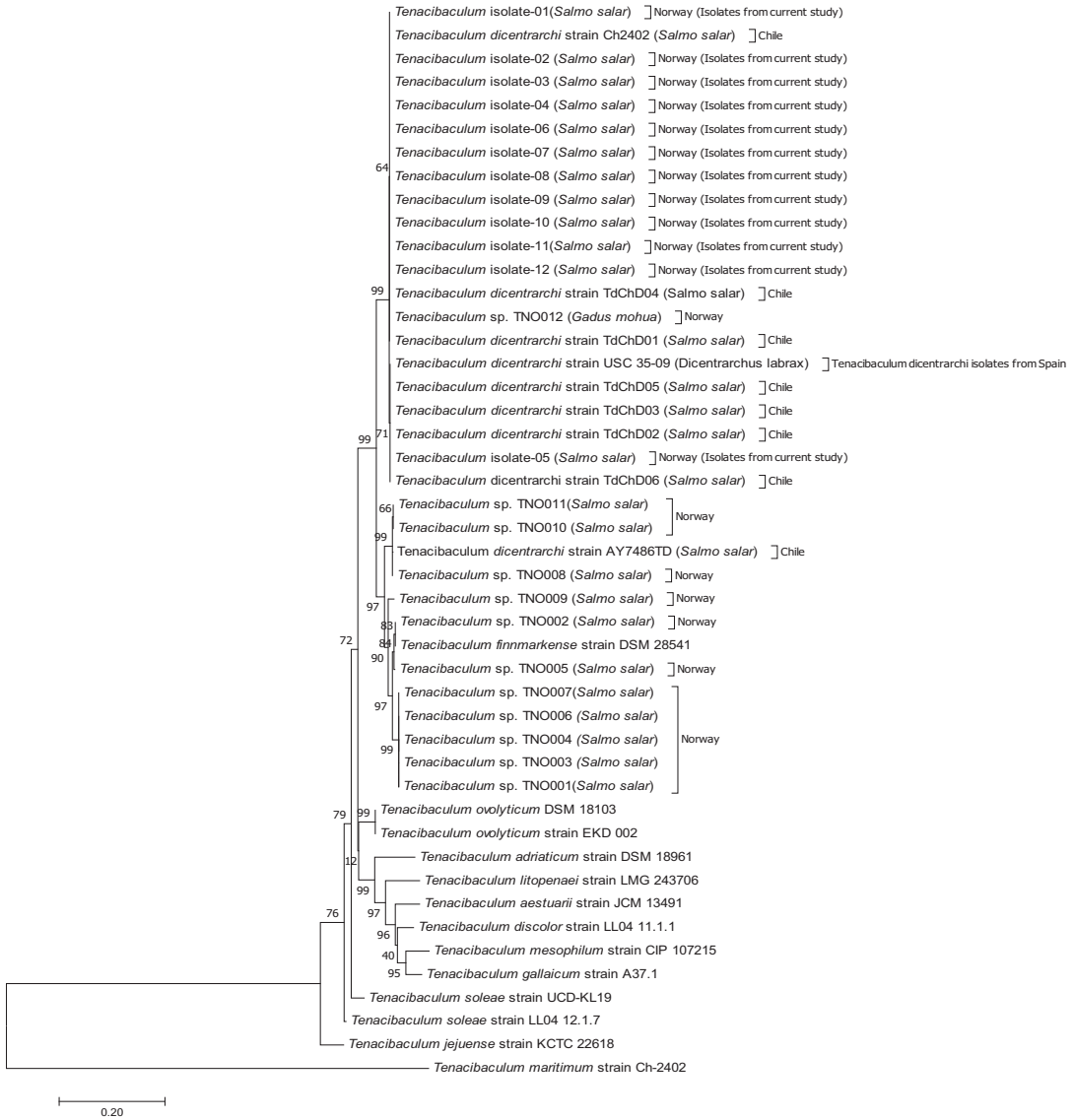

FIGURE A2 Evolutionary relationships of taxa based on *dnaK* gene. The evolutionary history was inferred using the neighbour-joining method. The optimal tree with the sum of branch length = 2.08589139 is shown. The confidence probability (multiplied by 100) that the interior branch length is greater than 0 as estimated using the bootstrap test (1,000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the maximum composite likelihood method and are in the units of the number of base substitutions per site. The differences in the composition bias among sequences were considered in evolutionary comparisons. The analysis involved 46 nucleotide sequences. Codon positions included were 1st+2nd+3rd+non-coding. All positions containing gaps and missing data were eliminated. There were a total of 490 positions in the final data set. Evolutionary analyses were conducted in MEGA7

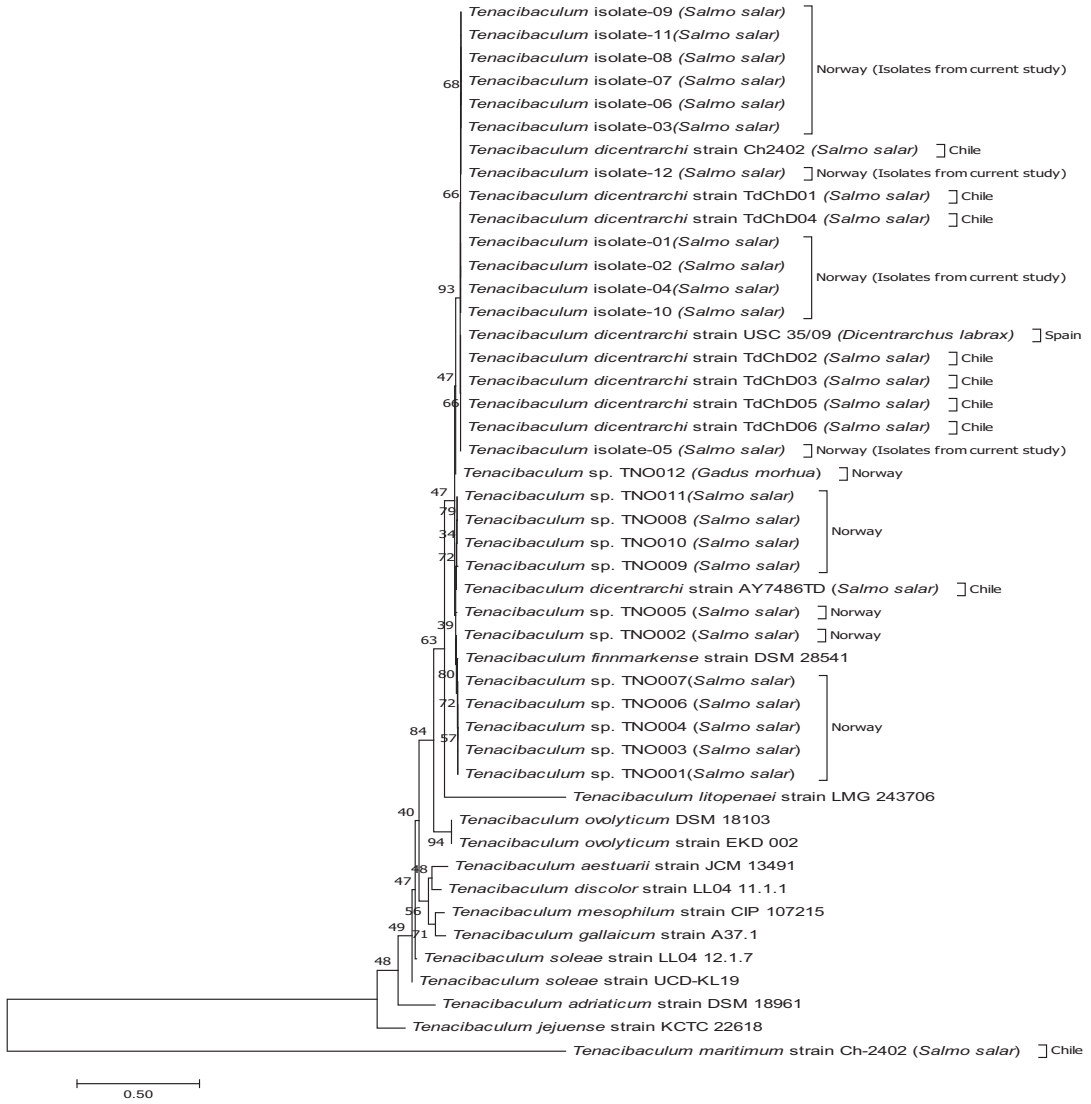

FIGURE A3 Evolutionary relationships of taxa based on *glyA* gene. The evolutionary history was inferred using the neighbour-joining method. The optimal tree with the sum of branch length = 5.25997874 is shown. The confidence probability (multiplied by 100) that the interior branch length is greater than 0 as estimated using the bootstrap test (1,000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the maximum composite likelihood method [4] and are in the units of the number of base substitutions per site. The differences in the composition bias among sequences were considered in evolutionary comparisons. The analysis involved 46 nucleotide sequences. Codon positions included were 1st+2nd+3rd+non-coding. All positions containing gaps and missing data were eliminated. There were a total of 542 positions in the final data set. Evolutionary analyses were conducted in MEGA7

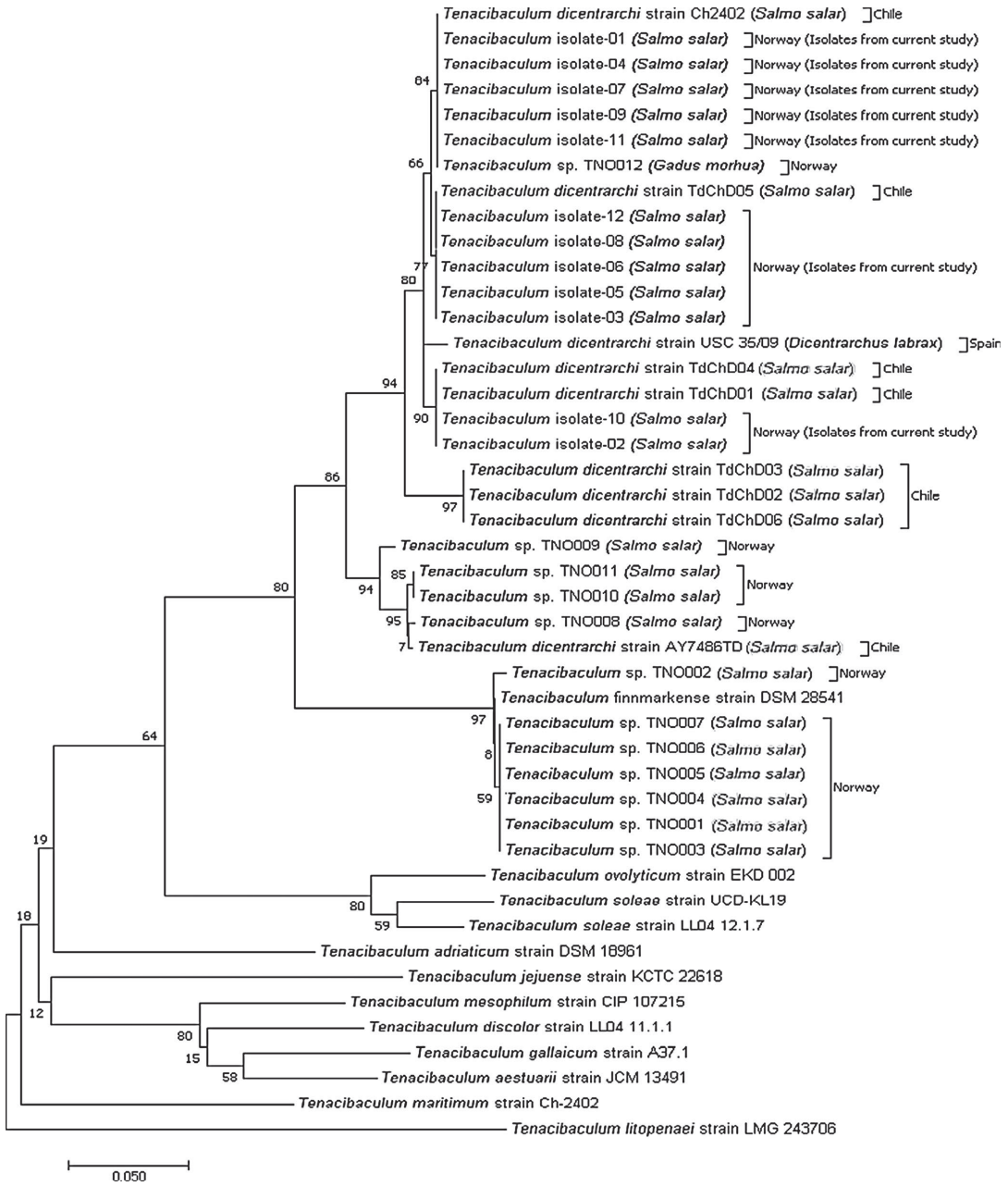

FIGURE A4 Evolutionary relationships of taxa based on *gyrB* gene. The evolutionary history was inferred using the neighbour-joining method. The optimal tree with the sum of branch length = 1.47657019 is shown. The confidence probability (multiplied by 100) that the interior branch length is greater than 0 as estimated using the bootstrap test (1,000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the maximum composite likelihood method and are in the units of the number of base substitutions per site. The differences in the composition bias among sequences were considered in evolutionary comparisons. The analysis involved 45 nucleotide sequences. Codon positions included were 1st+2nd+3rd+non-coding. All positions containing gaps and missing data were eliminated. There were a total of 407 positions in the final data set. Evolutionary analyses were conducted in MEGA7

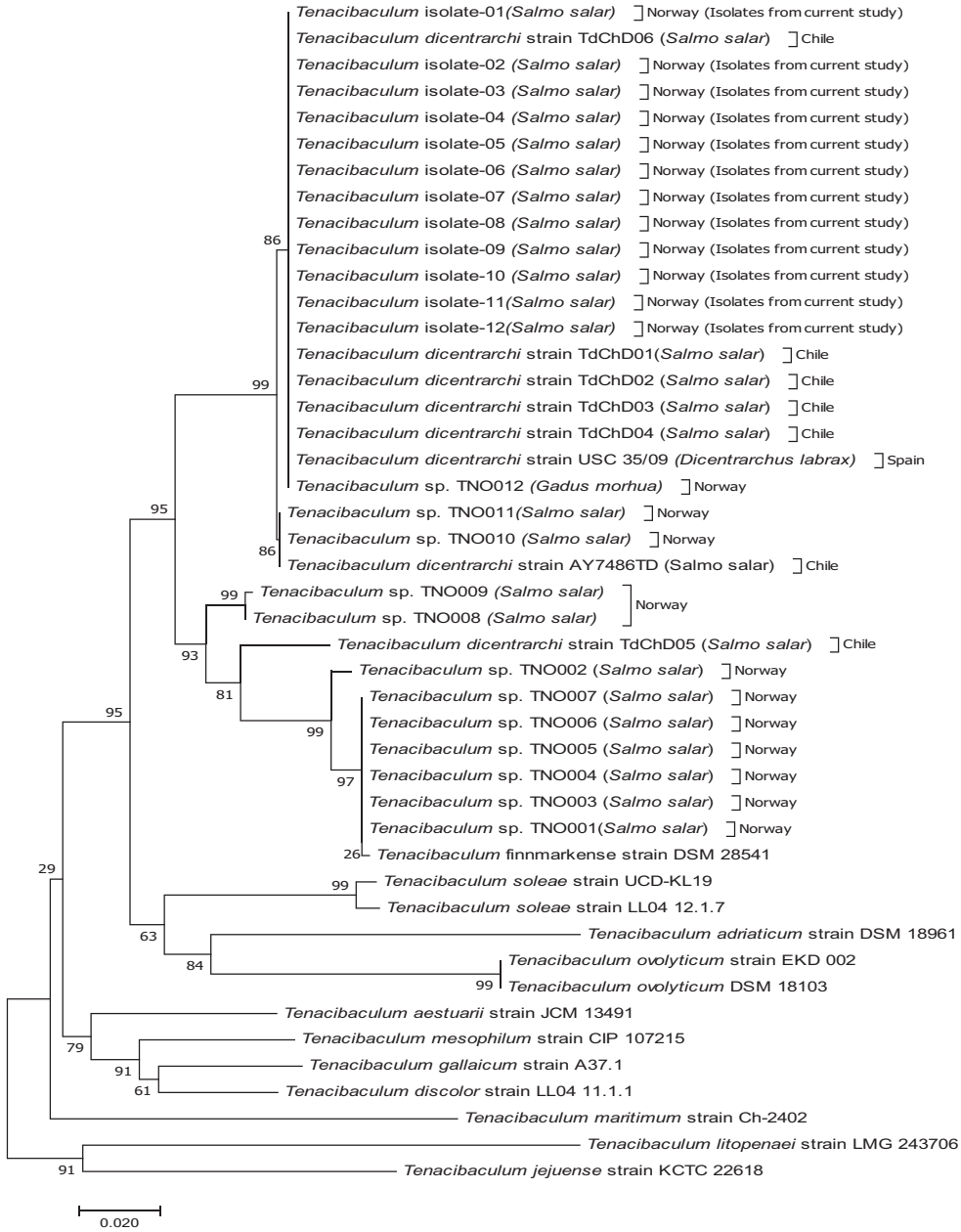

FIGURE A5 Evolutionary relationships of taxa based on *infB* gene. The evolutionary history was inferred using the neighbour-joining method. The optimal tree with the sum of branch length = 0.88763066 is shown. The confidence probability (multiplied by 100) that the interior branch length is greater than 0 as estimated using the bootstrap test (1,000 replicates) is shown next to the branches. The tree is drawn to scale, with branch length in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the maximum composite likelihood method and are in the units of the number of base substitutions per site. The differences in the composition bias among sequences were considered in evolutionary comparisons. The analysis involved 45 nucleotide sequences. Codon positions included were 1st+2nd+3rd+non-coding. All positions containing gaps and missing data were eliminated. There were a total of 564 positions in the final data set. Evolutionary analyses were conducted in MEGA7

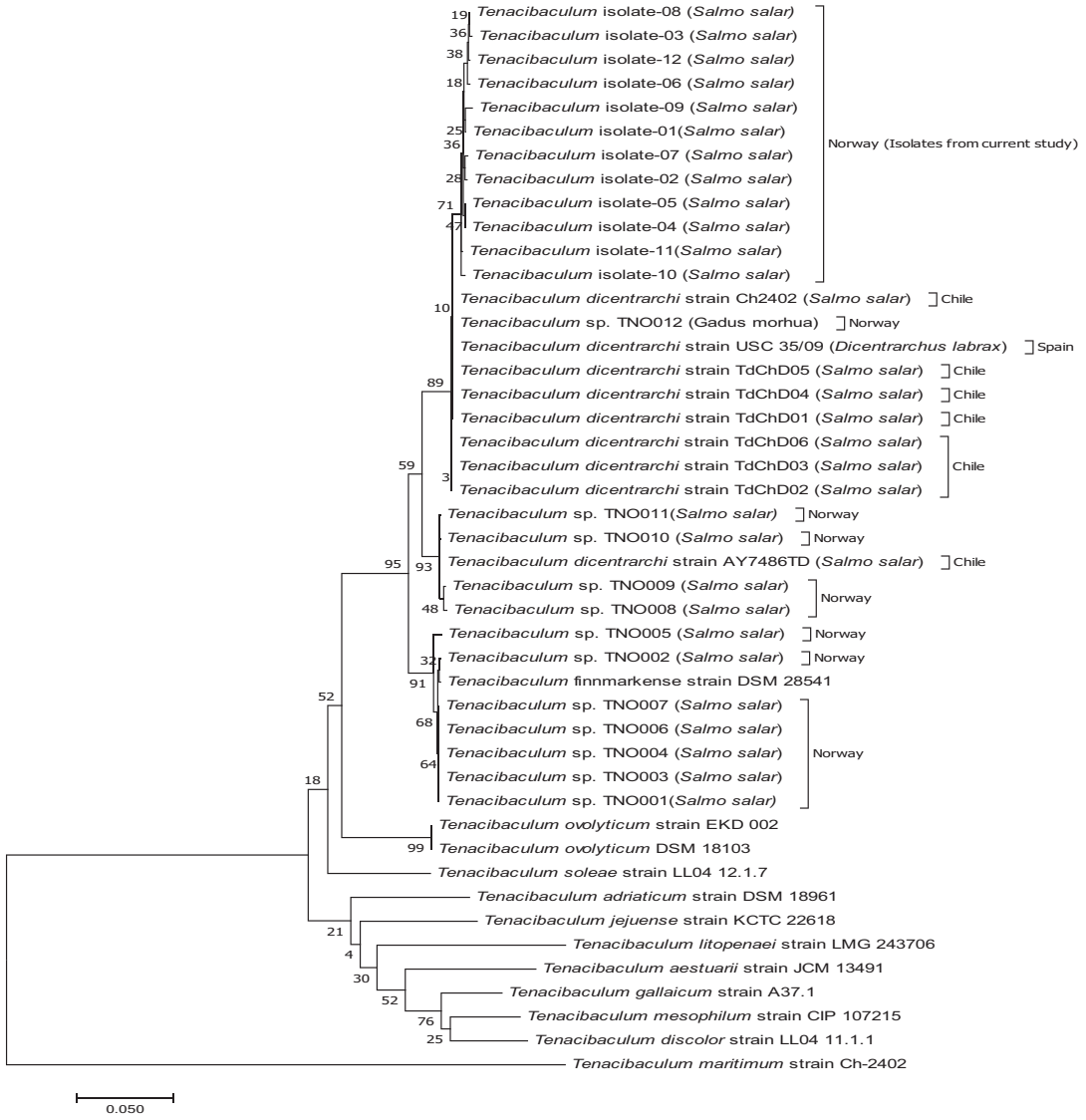

FIGURE A6 Evolutionary relationships of taxa based on *rlmN* gene. The evolutionary history was inferred using the neighbour-joining method. The optimal tree with the sum of branch length = 1.14129950 is shown. The confidence probability (multiplied by 100) that the interior branch length is greater than 0 as estimated using the bootstrap test (1,000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the maximum composite likelihood method and are in the units of the number of base substitutions per site. The differences in the composition bias among sequences were considered in evolutionary comparisons. The analysis involved 45 nucleotide sequences. Codon positions included were 1st+2nd+3rd+non-coding. All positions containing gaps and missing data were eliminated. There were a total of 483 positions in the final data set. Evolutionary analyses were conducted in MEGA7

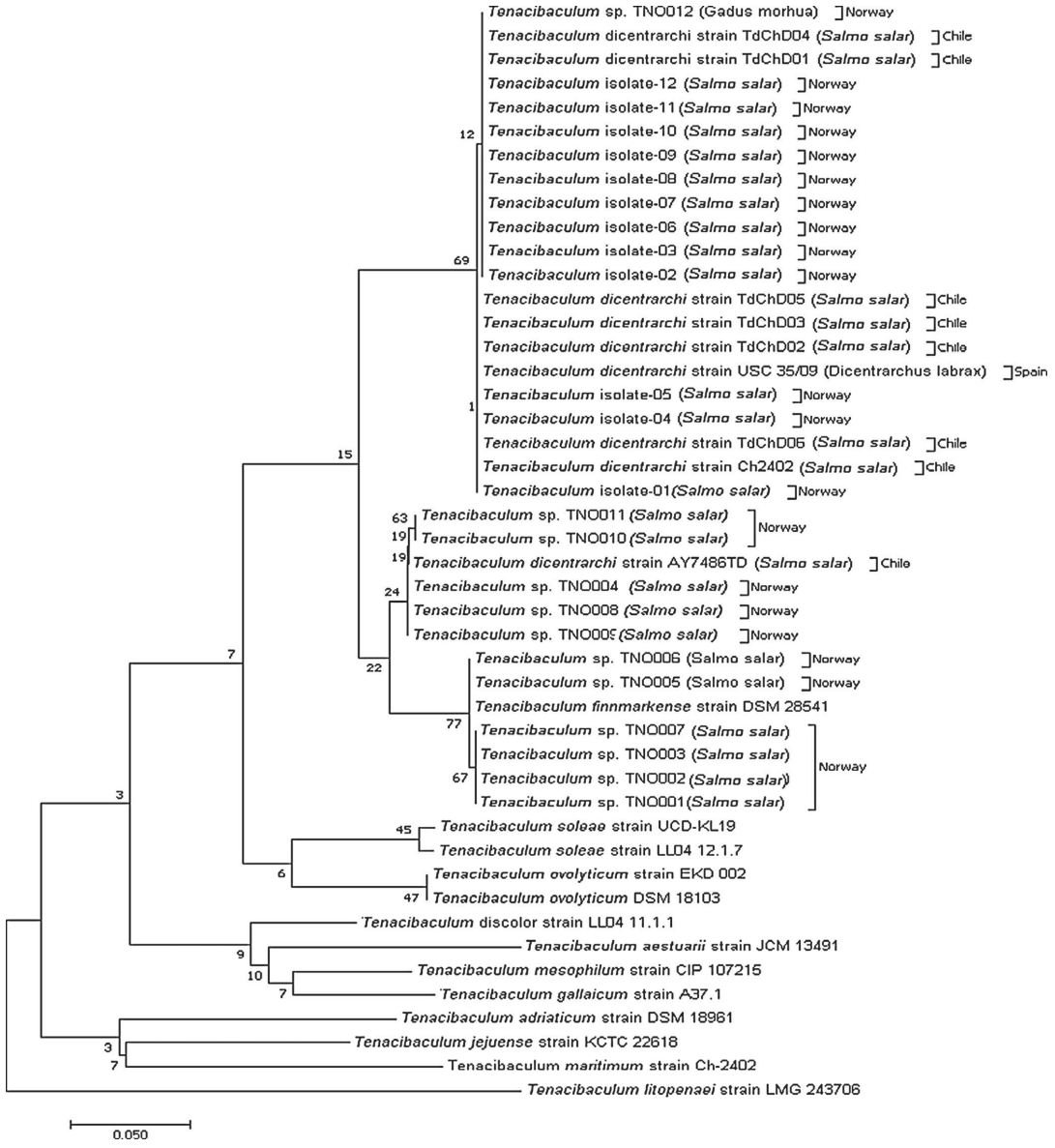

FIGURE A7 Evolutionary relationships of taxa based on *tgt* gene. The evolutionary history was inferred using the neighbour-joining method. The optimal tree with the sum of branch length = 1.31462876 is shown. The confidence probability (multiplied by 100) that the interior branch length is greater than 0 as estimated using the bootstrap test (1,000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the maximum composite likelihood method and are in the units of the number of base substitutions per site. The differences in the composition bias among sequences were considered in evolutionary comparisons. The analysis involved 46 nucleotide sequences. Codon positions included were 1st+2nd+3rd+non-coding. All positions containing gaps and missing data were eliminated. There were a total of 416 positions in the final data set. Evolutionary analyses were conducted in MEGA7

TABLE A1 Daily mortality first month after transfer. Mortality start to increase 6–9 days after transfer to the post-smolt plant (and sea water) reached top 10-16 days after transfer and decreased to almost normal after about 20 days

	Tank							
Date	E1	E2	E3	E4	F1	F2	F3	F4
01.Nov	Arrival	Arrival	Arrival					
02.Nov			Arrival	Arrival	Arrival	Arrival		
03.Nov	42					Arrival	Arrival	Arrival
04.Nov	2	36	68	16	34	32	21	38
05.Nov	3	3	7	8	10	8	9	6
06.Nov	8	2	4	5	6	4	2	6
07.Nov	7	2	8	3	3	9	0	2
08.Nov	54	3	6	3	6	2	5	8
09.Nov	218	8	19	7	3	3	3	5
10.Nov	323	28	121	7	6	3	0	7
11.Nov	127	47	203	62	32	34	6	11
12.Nov	191	35	134	59	59	84	9	9
13.Nov	352	26	194	90	75	153	51	25
14.Nov	234	52	669	817	148	531	195	7
15.Nov	357	18	265	657	48	86	128	40
16.Nov	460	26	317	1 066	38	126	235	69
17.Nov	113	18	131	1 011	29	66	133	40
18.Nov	109	24	51	504	24	54	103	17
19.Nov	87	10	76	535	21	72	63	8
20.Nov	49	12	52	388	33	191	109	21
21.Nov	246	30	48	296	50	101	50	20
22.Nov	46	29	19	61	63	136	55	4
23.Nov	23	8	12	117	36	149	14	4
24.Nov	30	8	9	81	31	63	9	4
25.Nov	17	10	14	38	54	57	8	0
26.Nov		3	4	27	16	27	0	0
27.Nov	23	0	0	20	20	21	0	3
28.Nov	4	17	17	20	39	50	5	1
29.Nov		6	4	6	10	25	1	
30.Nov		0			0			
Number of fish at start	77,376	77,176	74,041	77,722	79,022	75,000	77,629	73,443
Total number of dead	3,125	461	2,452	5,904	894	2,087	1,214	355
Percent dead	4.0	0.6	3.3	7.6	1.1	2.8	1.6	0.5

II

Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

SPECIAL ISSUE ARTICLE

Enhanced growth and decreased mortality in Atlantic salmon (*Salmo salar*) after probiotic bath

Ø. Klakegg[1,2], K. Salonius[1], A. Nilsen[3], M. Fülberth[4] and H. Sørum[2]

1 Previwo AS, Oslo, Norway
2 Faculty of Veterinary Medicine, Department of Food Safety and Infection Biology, Norwegian University of Life Sciences, Oslo, Norway
3 Norwegian Veterinary Institute, Oslo, Norway
4 Njord Salmon AS, Bioparken, Kjørsvikbugen, Norway

Keywords
Atlantic salmon, probiotic, health, growth, *Aliivibrio*.

Correspondence
Øystein Klakegg, Previwo AS, Ullevålsveien 68, Oslo, Norway.
E-mail: oystein.klakegg@previwo.no, post@previwo.no

2020/0211: received 3 October 2019, revised 13 March 2020 and accepted 21 March 2020

doi:10.1111/jam.14649

Abstract

Aims: Disease in farmed Atlantic salmon occurs in all its life stages. Salmon are particularly vulnerable to infectious diseases at transition from the freshwater stage to the saltwater stage. Our aim in these studies reported was to investigate the possibility that waterborne delivery of a probiotic comprised of naturally occurring marine bacterial species would reduce the mortality and improve the health and growth of farmed Atlantic salmon.
Methods and results: In three trials at two aquaculture production sites in Norway, isolates of *Aliivibrio* bacteria were added to the rearing water of Atlantic salmon. The fish were followed in 4–6 months after one single bath with observations and samplings. Growth, ulcers and survival were recorded. At the end of the studies growth was up to 31% larger in the probiotic enhanced groups and in trial 1 both mortality and prevalence of ulcer were significantly lower in the probiotic enhanced group compared to the control. Feed conversion rates were recorded in trial 1 and 2 and were from 9 to 28% better for the probiotic enhanced groups compared to the control groups.
Conclusion: Bathing of Atlantic salmon with probiotic *Aliivibrio* strains increased growth, reduced mortality and improved FCR in the postsmolt period.
Significance and Impact of the Study: The study demonstrates the potential to enhance growth, prevent ulcers and decrease mortality in Atlantic salmon after adding probiotic strains of *Aliivibrio* spp. into the rearing water. The study can have impact on animal welfare, economy and sustainability in the aquaculture industry.

Introduction

Since commercial fish farming started around 1970 in Norway, farming of Atlantic salmon (*Salmo salar*) has grown to be one of the most important industries.

The number of smolt put to sea in cages annually in Norway increased from 82·9 to 341·3 million from 1994 to 2018 (Norwegian Directorate of Fisheries). In the same period, the sale of slaughtered Atlantic salmon has increased from 204 686 metric tons round weight to 1 281 872 metric tons. The value of slaughtered Atlantic salmon has increased even more in the same period, due to a rising sales price per kg, from 5644 to 64 583 million NOK (Norwegian Fisheries Directorate). The production cost increased from 18·6 NOK per kg in 2008 to 30·7 NOK per kg in 2017 (Norwegian Directorate of Fisheries), and in the same period the economic feed conversion rate (eFCR) ranged between 1·21 and 1·35 (Norwegian Directorate of Fisheries). The year of 2017 was the second worst with an average eFCR at 1·32.

Efficient vaccination protocols were developed against cold water vibriosis (Lillehaug 1990) and furunculosis (Lillehaug *et al.* 1992), and the prevalence of these

diseases has been very low in Norway since the mid 1990s (Hjeltnes *et al.* 2018).

Most salmon in commercial Norwegian farms have been vaccinated with oil-based injection vaccines against *Aeromonas salmonicida* ssp *salmonicida, Aliivibrio (Vibrio) salmonicida* and *Vibrio anguillarum* since the mid-1990s. It is also common to vaccinate against *Moritella viscosa* (winter ulcer disease) and infectious pancreas necrosis virus. Vaccination against Salmonid Alphavirus has been performed in endemic areas, and vaccination against *Yersinia rückeri* has been used to mitigate increasing disease problems with enteric red mouth disease in hatcheries in certain regions of the coast. Preliminary trials with vaccination against *Tenacibaculum finnmarkense* are also reported (Småge *et al.* 2018). However, since the effective introduction of vaccines against bacterial infections in farmed salmonids in the 1980s and early 1990s betterment in survival has been nearly absent.

Despite continuous efforts to improve fish health, fish welfare and sustainability of the farming, the mortality, production cost and feed conversion rate (FCR) in Norwegian Atlantic salmon aquaculture production has not improved the last 20 years.

In the period from 1994 to 2018, annually accumulated losses from sea transfer to harvest ranged from 11·2% (1994) to 23·1% (2003). During the last 4 years the losses have varied between 15·8% (2018) and 17·8% (2016) (Norwegian Directorate of Fisheries). Epidemic bacterial and viral diseases have been important constraints to further growth of salmon production in all salmon producing countries (Asche *et al*. 2013). In Norway, high mortalities were associated with outbreaks of infectious salmon anaemia (Thorud and Djupvik 1988), cold water vibriosis (Egidius *et al.* 1986) and furunculosis (Grave and Brun 2016) prior to the mid-1990s.

There is no nationwide database of specific mortality causes. The few available reports and publications of mortality data, (Aunsmo *et al*. 2008; Bleie and Skrudland 2014; Takle *et al.* 2015) do not provide comparable data sets. In the annual reports from the Norwegian Veterinary Institute (NVI) and Institute for Marine Research (IMR), viral pathogens such as Salmon Alphavirus, infectious Salmon Anemia Virus (ISAV), Piscine Reovirus and Piscine Myocarditis Virus are listed as important causes of death. Other mortality causes are failed smolt, ulcers and fin rot and trauma connected to the different procedures involved in sea lice treatments (Grefsrud *et al.* 2018, Hjeltnes *et al.* 2019).

Ulcers and severe fin rot in farmed salmon during ongrowth in sea cages is associated with several bacterial pathogens, as *M. viscosa*, *Tenacibaculum* spp. and various *Vibrio* and *Aliivibrio* infections (Egidius *et al.* 1986; Lunder *et al.* 1995; Frans *et al.* 2011; Olsen *et al.* 2011; Karlsen *et al.* 2014; Småge *et al.* 2016; Klakegg *et al.* 2019). These bacterial infections with subsequent chronic ulcerative conditions lead not only to mortality, but also to impaired fish welfare to the survivors. Healed skin lesions is also an important cause of downgrading of harvested fish (Takle *et al.* 2015). Ulcers are responsible for more than 2·5% of salmon deaths during on-growth in sea-water, and also for more than 2·5% of the salmon being downgraded at the harvesting plant (Takle *et al.* 2015).

In addition to mortality and visible lesions, such bacterial infections also have a negative impact on metabolism and growth rates, induced by reduced appetite and increased metabolism caused by increased immune activity. Infections also cause stress and stress response faced with the acute challenge. Released catecholamines increase the heart rate and the cardiac output, providing more energy while less vital body functions as growth, reproduction and digestion are downregulated (Madaro *et al.* 2015; Vindas *et al.* 2016).

Probiotics are defined as live micro-organisms that confer several beneficial effects to the host, traditionally used as feed or feed additives to enhance the intestinal microbial balance (Balcazar *et al.* 2006). The first minor use of probiotics in aquaculture was done more than 30 years ago (Kozasa 1986). Competitive exclusion of other and less beneficial bacteria and immunomodulation of the host have been suggested as possible modes of action. Besides reduced growth of, or impact by, pathogenic bacteria, improved growth rates and feed conversion ratios are described as positive outcomes of probiotic applications (Merrifield *et al.* 2010; Martinez Cruz *et al.* 2012; Mohapatra *et al.* 2013; Banerjee and Ray 2017). The whole-body surface of fish (skin, gills and gut) is covered by a mucosal surface, in contrast to mammals where the gut mucosa is the principal target of probiotic application. In trials with *Aliivibrio salmonicida*, it has been shown that bacteria are taken up through the skin and are detected in the blood only minutes after application of bacteria to the rearing water (Kashulin and Sørum 2014). Application of probiotic bacteria by bath exposes all of the mucosal surfaces of fish including the skin to the probiotic bacteria, and a positive effect would not be limited to passing through and surviving the gut environment as is the case when application is through the feed. Thus, in fish there is a possibility that enhanced microbial balance could affect a wider range of immunological, physiological and metabolic processes. Although adding probiotic into feed is the most common way of application, bath treatments have also been tested with a wider range of both Gram positive and Gram negative bacteria (Newaj-Fyzul and Austin 2015). The observation that bacteria is taken up via intact skin in addition to the

gut system or gills, makes the introduction of probiotic bacteria to the fish much simpler. The fish receive the probiotic bacteria without eating and the bacteria do not need to be processed into feed and do not have to survive through the acidic stomach.

Probiotic bacteria have been applied to a variety of fish species. Several bacteria have been considered as candidates for use as probiotics also for Atlantic salmon, among them being: *Carnobacterium inhibens*, *Carnobacterium divergens, Lactobacillus delbrueckii, Pseudomonas fluorescens* and *Vibrio alginolyticus* (Austin *et al.* 1995; Gram *et al.* 1999; Irianto and Austin 2003; Ringø *et al.* 2007, Salinas *et al.* 2008).

Farmed lumpfish were bathed in probiotic *Aliivibrio* bacteria with promising results (Klakegg *et al.* 2020).

The main aim of this study was to examine the effect on growth, ulcer development, survival and feed conversion ratio in Atlantic salmon by bath applications with one, two or three different strains of *Aliivibrio* spp. and to examine if these potential effects depend on the concentration of the probiotic bacteria added to the rearing water.

Materials and Methods

This study was conducted as three individual trials with similar methods but different concentrations, duration of application and mixes of bacteria (Table 1). The rationale for the first trial to be conducted was to find alternative or complementary solutions to lessen the aquaculture site's large problems with prevalence of ulcers in vaccinated fish, probably caused by pathogenic bacteria as *M. viscosa* and *Aliivibrio wodanis*, and to test a reasoned hypothesis that adding probiotic bacteria to the rearing water would diminish the problem.

Material

Atlantic salmon

The Atlantic salmon in each one of the three trials were from the same batch, but their origin, strain and vaccine status varied between each trial (Table 1). The salmon had not been exposed to seawater before the time of application of probiotic bacteria. The salmon in trial 3 were pit-tagged with 12 mm RFID, itag 162 and the pit-tagging was done under benzocaine (Benzoak Vet. Anesthesia) (30 mg l^{-1})). Pit tags used were ISO 11784 and 11785 approved, and IEC 8-2-6/29 tested. Pit tags were used as recommended from dealer, BTS-ID. Pit tags were injected with a N125 needle, 12 days and more before start of trial.

Prior to application of the probiotic bacteria and entry into the trial, the general health status of the fish population was assessed and screened for known diseases. Only fish groups of good health status and with no known diseases were entered into the trials. The fish in trial 1 and 2 were followed by a veterinary health certificate and the fish in trial 3 were under surveillance by a fish health manager.

Bacteria

In trial 1, 2 and 3 combinations of *Aliivibrio* sp.,Vl1, NCIMB 42593 isolated from the mandibulum of farmed Atlantic salmon, *Aliivibrio* sp., Vl2, NCIMB 42592 isolated from the head kidney of farmed Atlantic salmon, and *Aliivibrio* sp., Vl3, NCIMB 42594 also isolated from the head kidney of farmed Atlantic salmon were applied. The bacteria are included in Patent Application PCT/EP2017/067169.

The bacteria were primarily cultivated on blood agar plate (OXOID, CM0271, blood agar base No. 2, with 5% bovine blood and 2·5% (w/v) NaCl at 10°C for 2–4 days. Then plated secondary in monoculture with the same type of blood agar plates and cultured at 10°C for 2–4 days. Later the cultivated monocultures were suspended in freeze broth (Luria–Bertani broth with 2·5% NaCl and glycerol 20%) and stored at −80°C.

Water, tanks, feed and time of bacterial enhancement

Tank volumes, feeding, disinfection of water and the water temperature in the trial were according to Table 2. In trial 1 and 2 feed was weighed. Water temperature was steady during the trials, and the average retention time of the water in each tank was 45–60 min. In trial 3, the individually PIT-tagged fish in each of the eight groups were housed separately in 180-liter tanks for 26 days after probiotic enhancement, then they were stocked together into one large tank (4 m^3).

In trial 1 and 2, the bacterial enhancement was done the day the salmon met seawater for the first time. In trial 3, the bacterial enhancement was performed 57 days before transfer to seawater. Seawater was added to obtain a salinity of approximately 15 ppt 57 days after the probiotic bath, and salinity was then increased to >25 ppt 18 days later.

No side effects during probiotic enhancement were noticed. After adding probiotic bacteria, the visual impression of fish behaviour was that the fish schooled homogenously with no sign of stress, like erratic swimming, jumping or sudden burst of speed.

Methods

Cultivating probiotic bacteria

The probiotic cultures were made by inoculating the bacteria from the stock of frozen culture broth on blood

Table 1 Overview over the trials

Trial	Weight start W0 (g)	Vaccine status	Smolt producer and strain	Bacterial strains	Concentration	Duration of treatment (min)	Number of salmon at start
1.	110	Pentium Forte Plus and an autogenic Red Mouth Disease vaccine	Nordheim, Aure	VI1, VI2 and VI3	1/360	60	4200
			Mowi	Control			4200
2.	88	AJ Micro	Belsvika, Hemne	Control			4000
				VI 3	1/464	60	4000
			Salmobreed	VI1, VI2 and VI3	1/325	60	4000
				VI1 and VI2	1/448	60	4000
3.	55	Nonvaccinated	Sørsmolt, Sannidal	VI1 and VI2	1/100	30	100
			Mowi	VI1 and VI2	1/600	30	100
				VI1 and VI2	1/1000	30	100
				VI2 and VI3	1/100	30	100
				VI2 and VI3	1/600	30	100
				VI2 and VI3	1/1000	30	100
				Control			200

Table 2 Tank volume, feeding, disinfections, type of water and water temperature in the trials

Trial	Tank volume (m3)	Different group in same or separate tanks	Feed	Water disinfection	Temperature (C°)	Seawater or freshwater after treatment
1.	150	Separate	Skretting Spirit, Automatic	UV, 120–140 mW cm^{-1}	14	Seawater
2.	150	Separate	Skretting Spirit, Automatic	UV, 120–140 mW cm^{-2}	14	Seawater
3.	0·18* and 4	Separate first then same	Handfed	UV, but probably inadequate due to lack of filtration of water before UV-treatment	8	100% freshwater the first 57 days, then gradually in 30 days more seawater until 100%†

*The fish were kept in small tanks for 26 days after treatment, then pooled into one larger tank.
†Due to an outbreak of *Moritella viscosa* the fish were treated with freshwater for a few days.

agar plate (OXOID, CM0271, blood agar base No. 2, with 5% bovine blood and 2·5% (w/v) NaCl) incubated at 12°C for 2–4 days. From the blood agar plates the bacteria were inoculated and cultivated further in liquid broth (Luria–Bertani broth with 2·5% NaCl) at 12°C for 2–4 days with shaking at 220 rev min^{-1}, by adding fresh liquid broth stepwise. The bacteria could be cultivated as monocultures and distributed to the rearing water directly from monoculture-broth or the bacteria were cultivated together during the last passage of cultivation and then distributed as multi (two or three) culture-broths to the rearing water. At the application times, samples were taken from the bacterial culture and diluted (10^2, 10^4, 10^6) in phosphate buffered saline (2·5%) phosphate buffered saline (PBS), and 100 μl was spread with an L-spreader on blood agar plates at 12°C. Colony forming units (CFU) were counted, usually after 2 days. The number of CFU at harvest was around 3×10^9 CFU per ml.

Bathing procedures

In trials 1 and 2, the fish were pumped directly into 150 m^3 circular tanks indoors, containing 500–1000 l of seawater to protect the fish against trauma while they

were pumped into the tanks. After the fish and the accompanying fresh water from the shipping were transferred, more seawater was added to a salinity of approximately 25 ppt. The enhancement volumes in the different tanks varied from 8·6 to 13 m^3. During the process, 24–40 l of probiotic cultures were added to the tanks, with effective dilutions from 1/325 to 1/464 (Table 1, Fig. 1). When combined, all the bacterial strains were in a 0·5:0·5 or 0·33:0·33:0·33 volume ratio. Sixty minutes after adding the bacteria to the tanks, the water flow was returned. During the bathing operation, the water was oxygenated and maintained at 80–100% saturation. The control fish received no probiotic bacteria and no placebo and were pumped directly into the final rearing units containing about 75 m^3 of seawater.

In trial 3, the water level (freshwater) of the tanks was lowered to 90 l, then 89 l of sea water was added, and the probiotic culture was added (0·18, 0·3 or 1·8 l). In the tank with the control group there was added 0·9 L of PBS. After 30 min, the water (freshwater) was turned on again. During the bathing, the water was oxygenated and maintained at 90–100% saturation.

Trial 3 was a blinded trial. The salmon and tanks were randomly selected for the treatment and people not involved in the daily managing of fish or analysis of the data performed the dip or bath applications.

Anaesthesia and euthanasia

Before weighing and scoring in trials and pit-tagging in trial 3, the salmon were anesthetized in a benzocaine bath (30 mg l^{-1}) (Benzoak®).

Before external scoring, weighing and necropsy in trial 1 and 2 and necropsy and end of trial in trial 3, the salmon were euthanized by an overdose of benzocaine. (300 mg l^{-1}).

Assessment of growth, ulcers, mortality and FCRs

The mortalities in all trials were reported daily.

At the end of trial 1, we used a circular (d 100 cm) net to catch 150 salmon from each tank. The net was lowered to the bottom of the tank and pulled up as quickly as possible by hand after about 1 min. The captured salmon were weighed (±1 g) and ulcers were registered. Seven days before the end of trial 1, 42 salmon from the bacteria enhanced tank and 37 salmon from the control tank were sampled by the same procedure and weighed.

The procedure was repeated at the end of trial 2, with a sample size of 59–61 salmon from each tank. The ulcers in trial 2 were scored in four categories: 0 = no ulcers; 1 = small, superficial ulcers not penetrating epidermis, no haemorrhage; 2 = larger ulcers, penetrating epidermis, sign of haemorrhage, 3 = severe ulcer penetrating muscle layer, haemorrhage or necrosis. In addition, fork length was measured (±0.5 cm).

Figure 1 Application of *Aliivibrio* spp. into a 150 m^3 tank in trial 2. Photo Henning Sørum. [Colour table can be viewed at wileyonlinelibrary.com]

The feeding in trial 1 and 2 was automatic, with rations determined by a combination of a feeding table and observed appetite. All the feed fed to the fish was weighed.

In trial 3 feed rations were determined from observed appetite during the last trial period, after mixing all fish into one tank.

Isolation of bacteria from fish

From trials 1 and 2, we cultivated bacteria from the ulcers, lesions and head kidneys of 10 salmon from each tank at each sampling point. From all observed ulcers, samples were inoculated on 2·5% blood agar and marine agar (Difco™ Marine Agar 2216) and incubated at 12°C. Samples from head kidney were inoculated on blood agar only.

In trial 3 samples were only taken from dead fish.

Antibodies against M. viscosa in trial 3

Blood sampling was performed 20 weeks postprobiotic application from 10 fish from each treated group and five

fish from the control group. After euthanasia by a benzocaine overdose, blood was collected from the caudal vein into heparinized vacutainer tubes. The plasma was separated by centrifugation and stored at −20°C until further analysis.

Antibody quantification by an enzyme-linked immunosorbent assay (ELISA) was performed as described earlier with minor modifications (Løvoll *et al.* 2009). *Moritella viscosa* cells were cultured in Luria Broth (2·5% salt) and washed three times in PBS. The resuspended pellet was kept on ice while being sonicated at least for 10 min. After protein determination the sonicated solution was used for coating the wells, 100 μl per well (5 μg protein ml^{-1}) in 0·05 mol l^{-1} carbonate buffer (pH 9·6) and incubated overnight at two dilutions at 4°C, 1 : 50 and 1/100 with the positive and negative control sera at the same dilution.

After overnight incubation, the initial sample and reference incubation was followed by a monoclonal antibody against rainbow trout immunoglobulin, clone 4C10, that is known to cross-react with Atlantic salmon Ig. The internal positive plasma control used on all plates, was produced from a pool of fish immunized with a whole formalin inactivated *M. viscosa* strain. The absorbance was read spectrophotometrically at 450 nm and the mean absorbance of duplicate wells was used. The results were expressed as the ratio (Rel OD) between the absorbance of the sample wells relative to positive control pool at the same dilution (1/50 is reported).

Statistics and formulas

Calculations of mean, SD, SE, upper and lower 95% confidential interval of the mean and probabilities based on t-tests and Fisher's exact test, were all conducted in JMP® (JMP®, ver. (Pro 14). SAS Institute, Cary, NC, 1989–2019).

The prevalence of ulcers in the two groups in trial 1 was first tested with a chi-squared test where observed counts and expected counts were compared to assess the probability of an effect of group (control/test) on the outcome variable. Then we compared the ulcer prevalence with a logistic regression model with ulcer as a dichotomous outcome variable (0/1), group as a dichotomous predictor variable (control/test) and weight (g) as a continuous predictor variable.

In trial 2 we did a box-plot of weight, length and condition factors (CFs) or all tanks at both sample points (22·1, and 15·3). Four outliers were removed, one from A2 and three from A3. Then we compared weight, length and CF between the tanks at each of the two sampling points with a mixed linear regression model (ML) with test group as fixed predictor variable and tank as random predictor variable.

CF was calculated as: $CF = 100 \cdot (WL^{-3})$.

Specific growth rate (SGR) was calculated as: $SGR = 100 \times (\ln(W1) - \ln(W0))/(t1 - t0)$ where W1 and W0 are weights on days $t1$ and $t0$ respectively.

Feed conversion rate: FCR = The total weight of feed is divided by the net production (final weight minus starting weight of biomasses).

Results

Trial 1

The mean weight of the 42 probiotic bacterially enhanced salmon sampled 7 days before the end of the trial were 46% heavier than the mean weight of the 37 salmon from the control tank, 551 g vs 376 g ($P < 0·01$).

At the end of the trial, 122 days after the transfer to the postsmolt site, 150 fish from each tank were sampled by net, anaesthetized, weighed and assessed for ulcers. The probiotic bacterially enhanced (probiotic) group was 31% heavier (Table 3, Figs 2 and 3) and 47% fewer fish in the probiotic group had ulcers (Table 4). The weight was significantly higher and the number of ulcers was significantly lower in the probiotic groups ($P < 0·01$). The total number of dead fish in the two groups during the trial was 140 (3·3%) in the enhanced group and 188 (4·5%) in the control.

In the final logistic regression model, there was a significant increase of ulcer risk in the control group ($P < 0·001$), but no significant change in risk of ulcers across the observed weight intervals ($P = 0·79$).

The probiotic fish had a feed conversion ratio that was 28% better than the control fish (Table 5). SGR was 1·52 in the probiotic group and 1·30 in the control group (Table 5).

Trial 2

At the weight sampling on day 71, the probiotic fish were 15–22% larger than the control fish and at the weight sampling on day 125 they were 11–25% larger (Table 6, Fig. 4). Probiotic fish were significantly larger than the control group on both measuring dates ($P < 0·01$). CF in the control group at sample 1 was lower than the bacterial enhanced groups, but with no significant difference at the end of the trial (Table 6, Fig. 5) The group treated with Vl3 had the highest measured CF at end of the trial.

The different tanks were fed approximately equally until day 71. Then the feeding was adjusted after weighing of the fish. The FCR in groups bathed in probiotic bacteria was 9–16% better than in the control groups at end of the trial (day 125) (Table 5) and the SGR in the

Table 3 Weight at end of trial 1 and 3

Trial	Cage	N_0	N_1	W_0	W_1 Mean	SD_1	t-$Test_2$
1	A3 control	4200	149	110	534	146	
	A4 strain Vl1, Vl2 and Vl3	4200	150	110	700	172	$P < 0{\cdot}001$
3	A 1/1000 Strain Vl1 and Vl2	100	67	55	312·1	92	—
	B 1/100 Strain Vl1 and Vl2	100	73	55	320·5	91	—
	C 1/600 Strain Vl1 and Vl2	100	63	55	319·8	102	—
	D Control	100	67	55	307·5	101	
	E 1/1000 Strain Vl2 and Vl3	100	56	55	305·9	75	—
	F 1/600 Strain Vl2 and Vl3	100	51	55	323·6	10	—
	G Control	100	55	55	309·3	83	
	H 1/100 Strain Vl2 and Vl3	100	69	55	297·7	81	—

N_0 = number of fish stocked in each cage at start, W_0, W_1, N_1 = sample size end, W_0 = start weight, W_1 = final weight, SD_1 = Standard deviation of the mean, *t*-test if weight in each treatment group are larger than control in that trial; —, No significant difference ($P > 0{\cdot}05$).

probiotic enhanced groups were 5–11% larger than in the control group.

There was low mortality in trial 2, between 0·60 and 0·65% in the different groups, and few fish with ulcers in the trial, between 0 and 1·7% in the different groups.

Trial 3

These fish were affected by an outbreak of disease caused by *M. viscosa* few days after the salinity was raised to 25 ppt. For animal welfare reasons, the water supply to the tanks was switched to freshwater for 1 week, 8 weeks after the rise to 25 ppt, to heal the ulcers caused by *M. viscosa*.

At the end of the trial, there were no significant differences between the mean weights of the different treatments (Table 3).

The mortality varied between the groups from 21% in group B (1/100 strain Vl1 and Vl2) to 44% in group F (1/600 strains Vl2 and Vl3). The 1/100 groups, both B (strain Vl1 and Vl2) ($P < 0{\cdot}01$) and H (strains Vl2 and Vl3) ($P < 0{\cdot}05$), had significantly less mortality than the pooled control (Table 7).

The anti-*M. viscosa* response measured as the antibody level against *M. viscosa* was inversely proportional to the concentration of the probiotic bath and thus to mortality and level of infection in the trial and not correlated to protection. The mean of the level of antibody measured by the relative OD was not significantly different between the groups but means differ from 0·37 in the 1/100 group to 0·67 in the control group (Fig. 6).

Discussion

Our results, particularly in trial 1 and 2 performed under close-to-field conditions, indicate that a single bath introduction of one or more of the three *Aliivibrio* spp. strains resulted in a higher growth rate in the salmon in the postsmolt phase that is, the first 3-4 months after sea transfer. The differently probiotic bacterially enhanced groups were from 11 to 31% larger than their respective controls 4 months after sea transfer.

This occurred despite the fact that the probiotic bacteria enhanced groups were subject to handling and intermediate storage during the bathing in a static water volume at higher density compared to the control groups at sea transfer, which has been shown to result in an increase in the stress hormones as cortisol and catecholamines (Tort 2011).

FCRs (Trials 1,2) improved in the probiotic enhanced groups. In trial 1, the FCR was as low as 0·73, which is very good compared to an industry average FCR around 1·0 and is probably quite close to the limit of the potential of the farmed Atlantic salmon today. In 2015, the best site in Norway had an FCR of 0·67 according to the Norwegian Directorate of Fisheries profitability survey of the production of Atlantic salmon (Norwegian Directorate of Fisheries). We are not aware of other studies reporting an improved FCR in farmed fish after probiotic bath or dip application. However, our results are similar to previous reports where lower FCRs and higher protein efficiency rates were observed after dietary supplementation of probiotics, for instance with *Bacillus* spp. given to Caspian salmon (Aftabgard *et al.* 2018).

In trial 3, both groups bacterially enhanced with 1/100 concentration had significantly ($P < 0{\cdot}05$) lower mortality than the pooled controls. The mechanism behind the probiotic protection against the disease is not clear. Plasma concentrations of antibodies against *M. viscosa* from survivors in trial 3 were inversely proportional to the concentration of bacteria in the probiotic bath. This indicates that the probiotic protection against mortality from disease caused by *M. viscosa* is not based on an increased activity in the humoral immune defence. This is in contrast to other studies for instance with immunization of Atlantic salmon with experimental vaccine for protection against *M. viscosa*, where a positive correlation between survival rates and median antibody levels at challenge times was found (Guz 2003). Another study indicated that *M. viscosa* might have escape mechanisms

Figure 2 Fish sampling from trial 1 sampled 7 days before the end of trial. The *Aliivibrio* enhanced group are the row to the right, the control group the row to the left. The *Aliivibro* enhanced group was significantly larger ($P < 0{\cdot}01$). Photo Henning Sørum. [Colour table can be viewed at wileyonlinelibrary.com]

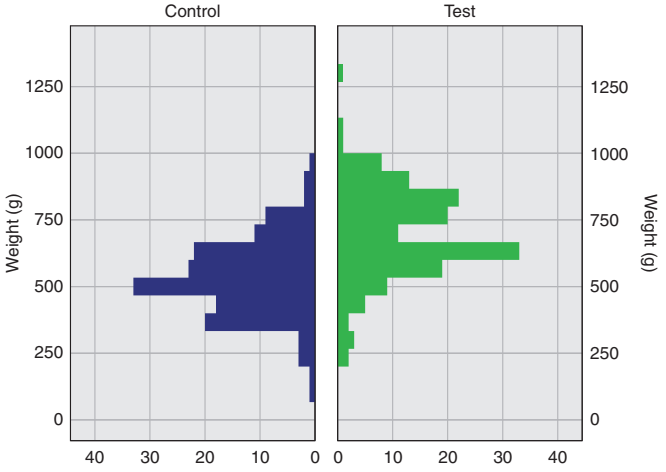

Figure 3 Weight distribution of fish at the end in trial 1. Number of fish at *x*-axis and weight at *y*-axis. [Colour table can be viewed at wiley onlinelibrary.com]

Table 4 Ulcer prevalence and mortality in trials 1 and 2 at the end of trials

Trial	Cage	*N*	N_w	Ulcer prevalence	Mortality (*n*)	Mortality (%)
1	A3 control	150	87	58·0	188	4·5
	A4 strain VI1, VI2 and VI3	150	46	30·7*	140*	3·3
2	A1 control	60	1	1·7	24	0·60
	A2 strain VI3	61	1	1·6	26	0·65
	A3 strain VI1, VI2 and VI3	61	1	1·6	24	0·60
	A4 strain VI1, and VI2	59	0	0·0	24	0·60

N = sample size, N_w = number of fish with wounds, Mortality = number of fish died during the trial, except sample fish.
*Significantly better than the control ($P < 0·05$) Fisher's exact test.

to evade the salmon immune system, this because the expression of the immune genes analysed in that study did not reveal increased expression until 7 days post challenge with *M. viscosa* (Løvoll *et al.* 2009). We suggest that the probiotic *Aliivibrio* spp. displace, dissuade or outcompete the *M. viscosa* before *M. viscosa* triggered the humoral immune system as has been reported as a mechanism of probiotic protective activity regarding other bacteria (Ibrahem 2015). Antibody response in Atlantic salmon has also been shown to correlate with the antigen dose from *A. salmonicida* ssp *salmonicida* (Romstad *et al.* 2013). In our trial, the antibody response in Atlantic salmon correlates with the degree of infection with *M. viscosa,* and since the degree of infection was lower in 1/100 bacterial enhanced groups it indicates that the probiotic *Aliivibrio* spp. somehow combat *M. viscosa* before *M. viscosa* triggered the humoral immune system. This would be particularly important in diseases with bacteria that have mechanisms to avoid the host immune response.

The results of trial 3 indicate that we should use a volume relation between the fermentation culture of bacteria and water larger than 1/600 or more than 5×10^6 CFU bacteria ml^{-1} water when bathing the fish to get positive

–>Feed conversion rate (FCR) and specific growth ratio (SGR) in trials 1 and 2

Table 5 Feed conversion rate (FCR) and specific growth ratio (SGR) in trials 1 and 2

Trial	Cage	N_0	W_{diff}	*t*	Feed	Net 5production	Mortality	FCR	SGR
1	A3 control	4200	424	122	1735	1701	188	1·02	1·30
	A4 strain VI1, VI2 and VI3	4200	590	122	1752	2395	140	0·73	1·52
2	A1 control	4000	551	136	2360	2123	$24_1 + 123_2$	1·11	1·46
	A2 strain VI3	4000	675	136	2620	2601	26 + 120	1·01	1·59
	A3 strain VI1, VI2 and VI3	4000	624	136	2407	2384	24 + 155	1·01	1·54
	A4 strain VI1 and VI2	4000	708	136	2546	2729	24 + 121	0·93	1·62

N_0 = number of fish stocked in each cage at start, W_{diff} = final weight (g)-start weight (g), *t* = number of days between start and end of the trial, Feed = kg feed given, Net production = total biomass increase from start to end, FCR = Feed conversion rate, Mortality = Number of dead fish ($_2$ = sampling and accident (A3) at transfer).

Table 6 Weights, lengths and CF in trial 2 at sampling day 71 (sample 1) and day 125 (sample 2). A2-4, is A2, A3 and A4 pooled.

		Sample 1							Sample 2						
			Weight (g)		Length		CF			Weight (g)		Length		CF	
Group	Tank	*n*	Mean	SD	Mean	SD	Mean	SD	*n*	Mean	SD	Mean	SD	Mean	SD
Control test	A1	63	271	50	30·5	2·0	0·94	0·06	60	639	122	35·5	2·0	1·39	0·14
	A2	59	326	60	31·5	1·5	1·01	0·07	60	763	158	37·0	3·0	1·48	0·13
	A3	58	312	66	31·5	2·0	1·01	0·07	60	712	132	38·0	2·5	1·30	0·09
	A4	62	331	69	31·5	2·0	1·02	0·08	59	796	172	39·0	2·5	1·31	0·08
	A2-4	179	323	65	31·5	2·0	1·01	0·07	179	757	758	38·0	2·5	1·36	0·13

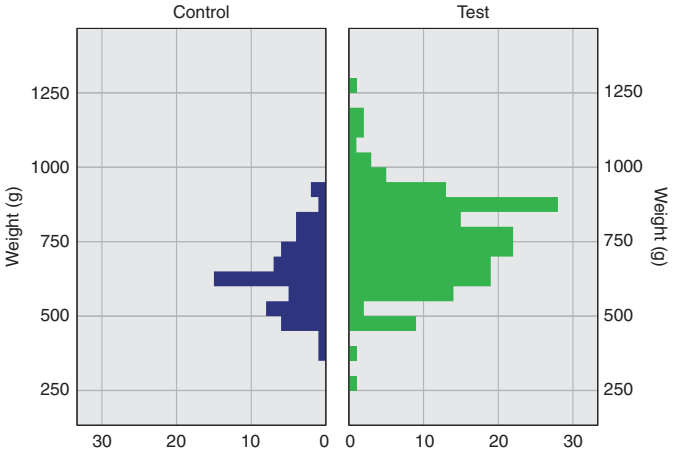

Figure 4 Weight distribution of fish at the end in trial 2. The test group is combined of data from all three tanks that were bacterial enhanced. Number of fish at *x*-axis and weight at *y*-axis. [Colour table can be viewed at wileyonlinelibrary.com]

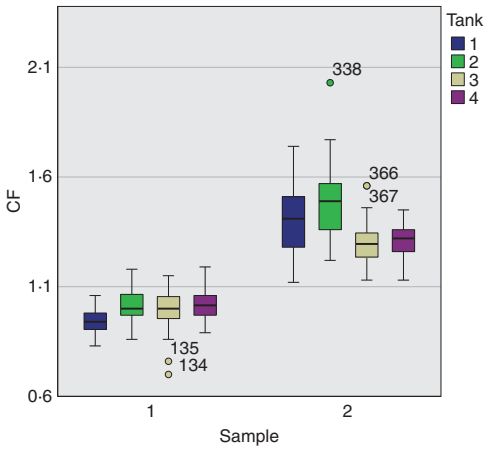

Figure 5 CF in trial 2 from weighing in day 71 (sample 1) and at the end of trial at day 125 (sample 2). All the bacterial enhanced groups were significantly heavier also at day 125, but tank 3 (Vl1, Vl2 and Vl3) and tank 4 (Vl1 and Vl2) had lower CF than the control group and tank 2 (Vl3) had larger CF than the control group at the end of trial (■ 1; ■ 2; ■ 3; ■ 4). [Colour figure can be viewed at wileyonlinelibrary.com]

effects. In trial 3, mortality rates were higher in the 1/600 concentration groups (both strains) than in the control groups. The relation between added culture of bacteria and water is not the only important factor. In trial 1 and 2, which gave the best results, we had a concentration between 1/325 and 1/464, much lower concentration than the 1/100 concentration we used in trial 3. In lumpfish bathed with the same bacteria (NCIMB 42592, NCIMB 42593) growth and survival was better when the lumpfish were bathed in a concentration of 2×10^7 CFU Aliivibrio spp ml^{-1} rearing water, while no positive effect was seen when the lumpfish bathed in a concentration of 7.5×10^5 CFU *Aliivibrio* ml^{-1} rearing water (Klakegg *et al.* 2020). It is likely that the probiotic bacteria contribute overall to the improved survival and growth of the Atlantic salmon, however, the contributive physiological effects on growth may have to do with metabolic cost of the infectious pressure as well as the bacterial concentration. Other factors like which species and the number of other bacteria that exist in the rearing water are most likely involved in this complex interplay, and what kind of challenges the fish meet may be of different importance from trial to trial depending on facility and water. The physiological status of the fish will also have an impact.

The smoltification process of the Atlantic salmon downregulates many immune genes involving different systems of the immune apparatus, among them, cytokines, signal transducers, antigen presentation, humoral and cellular innate immunity and T-cells (Johansson *et al.* 2016). It is also known that the number of mucus cells in the epidermis decrease to about 50% in connection with smoltification (O'Byrne-Ring *et al.* 2003). Salmon breeders also experience that the smolt often lose

Table 7 Mortality trial 3

Cage	*N*	N_d	N_a
A 1/1000 strain Vl1 and Vl2	100	31	69
B 1/100 strain Vl1 and Vl2	100	21	78*
C 1/600 strain Vl1 and Vl2	100	*32*	*65*
D Control	100	29	69
E 1/1000 strain Vl2 and Vl3	100	39	59
F 1/600 strain Vl2 and Vl3	100	44	54
G Control	100	38	60
H 1/100 strain Vl2 and Vl3	100	24	73*
Pooled 1/100 versus pooled control	200	45	151

Nd, Total number of dead fish. (Except 15 fish which died most probably after overdose of benzocaine after weighing. Also except nine fish that were dead, but did not have a pit-tag.) Na: total number of survivors.

*Significant ($P < 0{\cdot}05$ Fisher's exact test) better survival vs pooled control.

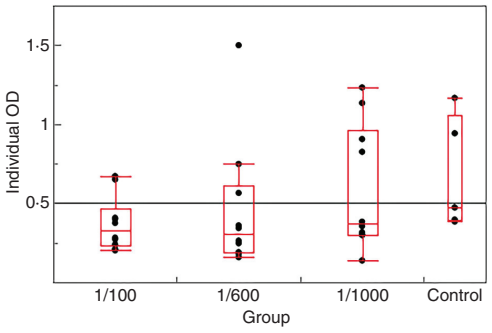

Figure 6 Variation and median antibody response (OD rel 1 : 50) against *Moritella viscosa* in fish bathed in probiotic *Aliivibrio* spp 1/100, 1/600 1/1000 and control. Each measurement is marked as: •. The mean was 0·372 for the 1/100 concentration, 0·456 for the 1/600 concentration, 0·590 for the 1/1000 concentration and 0·673 for the control. [Colour table can be viewed at wileyonlinelibrary.com]

many scales, and they often say that the 'scales are loose' in connection to the smoltification process. The transfer to the new saltwater environment presents as a risk in that the fish meet several new potential pathogens and while the immune system is downregulated with stress, the scales are loose and the mucus cells become fewer. Mortality, partly caused by infections has a peak incidence after sea transfer (Grefsrud *et al.*2018). The salmon appear to be more susceptible to bacterial invasion in that vulnerable period of life. Through long evolution it is unlikely that a downregulation of the immune system at this period would have been selected for if it was negative to the salmon life cycle. It is likely that the salmon makes itself more responsive for introduction of new bacteria it meets in the saltwater. Changes to the bacterial microbiome at this critical transition have been reported (Lokesh and Kiron 2016; Dehler *et al.* 2017). The importance of the bacterial microbiome to the salmon, and probably other fish, is much more profound than we know today.

Many of the bacterial species dominating the two habitats that Atlantic salmon migrate between, that is, freshwater and seawater, are taxonomically different, and the bacterial microbiome in saltwater and freshwater in Atlantic salmon is reported as clearly different (Llewellyn *et al.* 2016; Lokesh and Kiron 2016; Dehler *et al.* 2017). The *Aliivibrio* spp. we were bathing the fish with were originally cultivated from marine adapted salmon in seawater and we have shown that a purposeful and preparatory introduction of these bacteria have improved survival and growth.

The reason why we bathed the fish prior to transfer to seawater in trial 3 was a pilot trial that indicated better results regarding growth and survival when the fish was bathed prior to seawater transfer, compared with bathing at the time of transfer (data not shown), perhaps giving time for the probiotic bacteria to firmly establish as a part of the host microbiome. This supports the theory that establishment of a new bacterial microbiome is important to the salmon for the period of transition to seawater. When we give beneficial marine bacteria to the fish before they meet the potential pathogenic bacteria in the sea water, the fish will possibly be better equipped to contend with those pathogens. Still we saw less effect on growth in trial 3 bathed prior to transfer to seawater compared to trial 1 and 2 bathed at transfer to seawater. One reason could be that the time of nearly 2 months from application to transfer, reduced the proportion of the acquired marine *Aliivibrio* spp. to some extent before transferring to seawater. We think the Atlantic salmon have some probiotic marine bacteria, working best at seawater and other bacteria working best at the freshwater stage. An optimal time for seeding with probiotic bacteria to improve outcomes of the freshwater to marine environment transition is the subject of ongoing studies.

When we compare the three different strains of bacteria, it is shown that all three strains had positive effects on the fish. In trials 2 and 3 where we bathed with different strains, the bath with Vl1 and Vl2 performed best, regarding both growth and mortality. However, the results are not conclusive. When bathed with Vl3 alone in trial 2, the concentration was reduced compared to the other bath, and concentration matters (Hai 2015). The trials indicate that the strain effect is less than the effect of the overall concentration of the probiotic bacteria. An unexpected result was the calculation of CFs in trial 2,

that revealed that the fish in tank 3 and 4 from the first measurement at day 71 to the last measurement at day 125 grew fast but had a relatively lower CF than both the control and the Vl3 fish. Could it be that the bacteria Vl1 and Vl2 somehow made the fish to grow fast in length at that time of life, for later gain relatively more weight? This is speculative, but we know that bacteria in other species can affect anatomic development as reported of another *Aliivibrio*, *Aliivibrio fischeri*, that seems to influence the development of lens formation of the bobtail squid (*Euprymna scolopes*; Backhed 2019; Moriano-Gutierrez *et al.* 2019). In trial 1, where the fish in the probiotic group were 31% larger and had significant fewer ulcers than the control fish were bathed with all three *Aliivibrio* spp. In both trial 1 and 2 the fish in tank A4 was largest at end of the trials. The reason could be that there was some tank effect, but the site has not recognized this neither in fish groups before nor later in the same tanks. The control tank was not the same in trial 1 and 2 which also diminish the risk of tank effect.

Traditionally probiotics have mainly been associated with feed supplements and to act via the gastrointestinal tract (Merrifield *et al.* 2010; Nayak 2010).

There are some earlier reports from trials in which probiotic bacteria were added to the rearing water (Austin *et al.* 1995; Apun-Molina 2009). An intact mucus and skin in fish has earlier been considered as almost impenetrable for bacteria and the uptake of bacteria via the intact skin was considered as less important. However, in experiments bacteria quickly enter the fish and detected in the blood even when the bacteria are not exposed to the gills, mouth or gut (Kashulin and Sørum 2014). We have seen the same immediate uptake into Atlantic salmon of the three probiotic *Aliivibrio* employed in this study. After dipping the salmon in a bacterial bath, cultivating of the probiotic *Aliivibrio* strains was done from blood samples drawn 1 min after dipping (data not shown).

In a study, healthy lumpfish farmed as cleaner fish for controlling sea lice infestation in farmed Atlantic salmon were bathed with two (NCIMB 42592 and NCIMB 42593) of the three aliivibrio isolates used in the trials of this study. The probiotic exposed lumpfish had fewer ulcers and decreased mortality after a natural outbreak of disease caused by *M. viscosa* compared to the control groups. The growth also tended to be better in the probiotic enhanced groups (Klakegg *et al.* 2020).

Bath or dip application simplifies the application mode for probiotic bacteria. With administration to the rearing water, all mucosal surfaces are exposed to the probiotic bacteria. It is our hypothesis that the skin is one of the major routes of entry for the bacteria into the fish. After introduction, we speculate that cells of the innate immune system respond differently to the presence of probiotic bacteria with different cell signalling mechanisms and this leads to the retention and persistence of desirable or friendly bacteria. This will be the subject of future studies.

The intimate interactions between different bacteria in fish as in other species have been described and several ways of mechanism of actions are suggested that is, competitive exclusion of pathogenic micro-organisms, as a source of nutrients and enzymatic contribution to digestion, a way of improving the water quality, enhancement of the immune response or an antiviral effect (Merrifield *et al.* 2010; Martinez Cruz *et al.* 2012; Banerjee and Ray 2017).

In order to act positively on the fish, the probiotic bacteria should be recognized by the fish as harmless (Lazado and Caipang 2014). A possible mechanism could be that probiotic bacteria are recognized as non-pathogenic by the germline encoded pathogen pattern recognition receptors (PRRs) and that a cascade effect after contact with the PRRs will decide if the probiotic will result in symbiotic coexistence or if the fish will try to fight against the bacteria by exerting antibacterial effects (Lazado and Caipang 2014). This has so far been studied in the intestine. The bacterial extracellular communications with other bacteria with small molecules, the quorum sensing, provide a mechanism for bacteria to modulate their gene expression relative to other nearby bacteria (Camilli and Bassler 2006). *Moritella viscosa* and *Aliivibrio wodanis* are both pathogens and both often coexist in ulcers and internal organs in Atlantic salmon. It has been shown that that the presence of *A. wodanis* has an inhibiting effect on *M. viscosa*. (Hjerde *et al.* 2015). The probiotic *Aliivibrio* spp. used in these trials are phylogenetically close to *A. wodanis* (data not shown). It has been postulated earlier that the inhibiting effect of these *Aliivibrio* spp against *M. viscosa* could be the same as *A. wodanis*‘ inhibiting mechanisms towards *M. viscosa* (Klakegg *et al.* 2020). Many bacteria have inhibiting effects on other bacteria and some probably also promoting effects (Verschuere *et al.* 2000; Balcazar *et al.* 2006; Dimitroglou *et al.* 2011; Mohapatra *et al.* 2013; Akhter *et al.* 2015; Banerjee and Ray 2017). Until now, most researchers in the field believe that the inhibiting and promoting effects among bacteria are happening at mucosal surfaces, that is, skin, gut epithelium or gills. However, if bacteria are able to migrate through intact skin and into the blood in the fish (Kashulin and Sørum 2014), the modulating system of the holobiome is probably much more complex than we currently understand.

The documentation of immediate transfer of *A. salmonicida* across the skin of salmon to the blood vessels (Kashulin and Sørum 2014) and the unpublished observation that the three probiotic *Aliivibrio* isolates used in

this study are able to cross the skin is potentially fundamental to understand the results of the trials reported here. The physiological changes occurring in the smoltification process of Atlantic salmon just before meeting the marine water microbiome makes it interesting to speculate if the smolt is preparing for meeting and accepting the marine bacteria in the new environment. *Aliivibrio* bacteria are isolated from many different marine animal species for instance lumpfish which also harbour the same aliivibrio bacteria used as probiotic isolates in this study. These three independent observations mentioned make it natural to speculate that the interaction between bacteria and the fish host including salmon is much more wide, intimate and profound than what is accepted today.

The introduction of *Aliivibrio* spp. as a waterborne bath exposure to Atlantic salmon improved growth rates, with lower feed consumption rate, reduced mortality rates, reduced prevalence of ulcers and the ability to resist bacterial diseases better. We think that the probiotic bacteria act by inhibiting other bacteria, as indicated by the reduced level of infection and through reduced level of antibodies against *M. viscosa*. In addition there seems to be a potential probiotic effect on growth and FCRs by a direct effect on feed utilization.

In conclusion, bathing or dipping Atlantic salmon with probiotic *Aliivibrio* spp. indicated increased growth, improved FCR and reduced mortality and number of ulcers for a prolonged period after transfer to seawater.

Acknowledgements

We thank Liv Jorun Reitan who performed the antibody quantification against *M. viscosa*.

The work was supported by the Norwegian Research Council (grant no. 260204/O30).

Conflict of Interest

The principle author, Øystein Klakegg has been supported by the Norwegian research council (project number 260204) in an industrial PhD project headed by Previwo AS, Henning Sørum is one of the owners and founder of Previwo, the company that produces a probiotic product, Stembiont™ including the *Aliivibrio* strains under investigation in this report and Kira Salonius is the Managing Director in Previwo AS.

References

Aftabgard, M., Salarzadeh, A. and Mohseni, M. (2018) The effects of a synbiotic mixture of galacto-oligosaccharides and Bacillus strains in Caspian Salmon, *Salmo trutta* caspius Fingerlings. *Probiotics and Antimicrobial Proteins* **11**(4), 1300–1308. https://doi.org/10.1007/s12602-018-9498-4.

Akhter, N., Wu, B., Memon, A.M. and Mohsin, M. (2015) Probiotics and prebiotics associated with aquaculture: a review. *Fish Shellfish Immunol* **45**, 733–741. https://doi.org/10.1016/j.fsi.2015.05.038

Asche, F., Roll, K.H., Sandvold, H.N., Sorvig, A. and Zhang, D.J. (2013) Salmon aquaculture: larger companies and increased production. *Aquacult Econ Manag* **17**, 322–339. https://doi.org/10.1080/13657305.2013.812156

Aunsmo, A., Bruheim, T., Sandberg, M., Skjerve, E., Romstad, S. and Larssen, R.B. (2008) Methods for investigating patterns of mortality and quantifying cause-specific mortality in sea-farmed Atlantic salmon Salmo salar. *Dis Aquat Organ* **81**, 99–107. https://doi.org/10.3354/dao01954

Austin, B., Stuckey, L.F., Robertson, P.A.W., Effendi, I. and Griffith, D.R.W. (1995) A probiotic strain of *Vibrio alginolyticus* effective in reducing diseases caused by *Aeromonas salmonicida*, *Vibrio anguillarum* and *Vibrio ordalii*. *J Fish Dis* **18**(1), 93–96. https://doi.org/10.1111/j.1365-2761.1995.tb01271.x

Backhed, F. (2019) Shining light on microbial signaling to distant organs. *Proc Natl Acad Sci U S A* **116**(16), 7617–7619. https://doi.org/10.1073/pnas.1902664116

Balcazar, J.L., de Blas, I., Ruiz-Zarzuela, I., Cunningham, D., Vendrell, D. and Muzquiz, J.L. (2006) The role of probiotics in aquaculture. *Vet Microbiol* **114**, 173–186. https://doi.org/10.1016/j.vetmic.2006.01.009

Banerjee, G. and Ray, A.K. (2017) The advancement of probiotics research and its application in fish farming industries. *Res Vet Sci* **115**, 66–77. https://doi.org/10.1016/j.rvsc.2017.01.016

Bleie, H. and Skrudland, A. (2014) Tap av laksefisk i sjø. https://www.mattilsynet.no/fisk_og_akvakultur/fiskevelferd/tap_av_laksefisk_i_sjo_2014

Camilli, A. and Bassler, B.L. (2006) Bacterial small-molecule signaling pathways. *Science* **311**, 1113–1116. https://doi.org/10.1126/science.1121357

Dehler, C.E., Secombes, C.J. and Martin, S.A.M. (2017) Seawater transfer alters the intestinal microbiota profiles of Atlantic salmon (*Salmo salar* L.). *Sci Rep* **7**(1), 13877. https://doi.org/10.1038/s41598-017-13249-8

Dimitroglou, A., Merrifield, D.L., Carnevali, O., Picchietti, S., Avella, M., Daniels, C. and Davies, S.J. (2011) Microbial manipulations to improve fish health and production – a Mediterranean perspective. *Fish Shellfish Immunol* **30**, 1–16. https://doi.org/10.1016/j.fsi.2010.08.009

Egidius, E., Wiik, R., Andersen, K., Hoff, K.A. and Hjeltnes, B. (1986) *Vibrio salmonicida* sp. nov., a new fish pathogen. *Int J Syst Bacteriol* **36**, 518–520. https://doi.org/10.1099/00207713-36-4-518

Frans, I., Michiels, C.W., Bossier, P., Willems, K.A., Lievens, B. and Rediers, H. (2011) Vibrio anguillarum as a fish pathogen: virulence factors, diagnosis and prevention. *J Fish Dis* **34**, 643–661. https://doi.org/10.1111/j.1365-2761.2011.01279.x

Gram, L., Melchiorsen, J., Spanggaard, B., Huber, I. and Nielsen, T.F. (1999) Inhibition of vibrio anguillarum by *Pseudomonas fluorescens* AH2, a possible probiotic treatment of fish. *Appl Environ Microbiol* **65**, 969–973.

Grave, K. and Brun, E. (2016) Use of antibiotics in Norwegian aquaculture. Norwegian Veterinary Institute. https://www.vetinst.no/rapporter-og-publikasjoner/rapporter/2016/use-of-antibiotics-in-norwegianaquaculture

Grefsrud, E.S., Glover, K., Grøsvik, B.E., Husa, V., Karlsen, Ø., Kristiansen, T. and Svåsand, T. (2018) *Risikorapport norsk fiskeoppdrett 2018*. Bergen: Institute of Marine Research Retrieved from: https://www.imr.no/filarkiv/2018/02/risikorapport_2018.pdf/nn-no

Guz, L. (2003) Humoral antibody response of atlantic salmon, *Salmo salar* L., vaccinated against *Moritella viscosa*. *Pol J Vet Sci* **6**(3 suppl), 15–17.

Hai, N.V. (2015) The use of probiotics in aquaculture. *J Appl Microbiol* **119**, 917–935. https://doi.org/10.1111/jam.12886

Hjeltnes, B., Bang-Jensen, B., Bornø, G., Haukaas, A. and Walde, C.S. (2018) *The Health Situation in Norwegian Aquaculture 2018*. Oslo: Norwegian Veterinary Institute Retrieved from: https://www.vetinst.no/rapporter-ogpublikasjoner/rapporter/2018/fish-health-report-2017

Hjeltnes, B., Bang Jensen, B., Bornø, G., Haukaas, A. and Walde, C.S. (2019) *The Health Situation in Norwegian Aquaculture 2018*. Oslo: Norwegian Veterinary Institute Retrieved from https://www.vetinst.no/rapporter-ogpublikasjoner/rapporter/2019/fiskehelserapporten-2018

Hjerde, E., Karlsen, C., Sørum, H., Parkhill, J., Willassen, N.P. and Thomson, N.R. (2015) Cocultivation and transcriptome sequencing of two co-existing fish pathogens Moritella viscosa and *Aliivibrio wodanis*. *BMC Genom* **16**, 447. https://doi.org/10.1186/s12864-015-1669-z

Ibrahem, M.D. (2015) Evolution of probiotics in aquatic world: potential effects, the current status in Egypt and recent prospectives. *J Adv Res* **6**, 765–791. https://doi.org/10.1016/j.jare.2013.12.004

Irianto, A. and Austin, B. (2003) Use of dead probiotic cells to control furunculosis in rainbow trout, *Oncorhynchus mykiss* (Walbaum). *J Fish Dis* **26**, 59–62.

Johansson, L.H., Timmerhaus, G., Afanasyev, S., Jorgensen, S.M. and Krasnov, A. (2016) Smoltification and seawater transfer of Atlantic salmon (*Salmo salar* L.) is associated with systemic repression of the immune transcriptome. *Fish Shellfish Immunol* **58**, 33–41. https://doi.org/10.1016/j.fsi.2016.09.026

Karlsen, C., Vanberg, C., Mikkelsen, H. and Sørum, H. (2014) Co-infection of Atlantic salmon (*Salmo salar*), by *Moritella viscosa* and *Aliivibrio wodanis*, development of disease and host colonization. *Vet Microbiol* **171**, 112–121. https://doi.org/10.1016/j.vetmic.2014.03.011

Kashulin, A. and Sørum, H. (2014) A novel in vivo model for rapid evaluation of *Aliivibrio salmonicida* infectivity in Atlantic salmon. *Aquaculture* **420**, 112–118. https://doi.org/10.1016/j.aquaculture.2013.10.025

Klakegg, Ø., Abayneh, T., Fauske, A.K., Fulberth, M. and Sørum, H. (2019) An outbreak of acute disease and mortality in Atlantic salmon (*Salmo salar*) post-smolts in Norway caused by *Tenacibaculum dicentrarchi*. *J Fish Dis* **42**, 789–807. https://doi.org/10.1111/jfd.12982

Klakegg, Ø., Myhren, S., Juell, R.A., Aase, M., Salonius, K. and Sørum, H. (2020) Improved health and better survival of farmed lumpfish (*Cyclopterus lumpus*) after a probiotic bath with two probiotic strains of Aliivibrio. *Aquaculture* **518**, 734810. https://doi.org/10.1016/j.aquaculture.2019.734810

Kozasa, M. (1986) Toyocerin (*Bacillus toyoi*) as growth promotor for animal feeding. *Microbiol Alim Nutr* **4**, 121–124.

Lazado, C.C. and Caipang, C.M. (2014) Mucosal immunity and probiotics in fish. *Fish Shellfish Immunol* **39**, 78–89. https://doi.org/10.1016/j.fsi.2014.04.015

Llewellyn, M.S., McGinnity, P., Dionne, M., Letourneau, J., Thonier, F., Carvalho, G.R., Derome, N. (2016) The biogeography of the atlantic salmon (*Salmo salar*) gut microbiome. *ISME J* **10**(5), 1280–1284. https://doi.org/10.1038/ismej.2015.189

Lillehaug, A. (1990) A field trial of vaccination against cold-water vibriosis in Atlantic Salmon (*Salmo salar* L). *Aquaculture* **84**(1), 1–12. https://doi.org/10.1016/0044-8486(90)90295-X

Lillehaug, A., Lunder, T. and Poppe, T.T. (1992) Field testing of adjuvanted furunculosis vaccines in Atlantic Salmon, *Salmo salar* L. *J Fish Dis* **15**, 485–496. https://doi.org/10.1111/j.1365-2761.1992.tb00680.x

Lokesh, J. and Kiron, V. (2016) Transition from freshwater to seawater reshapes the skin-associated microbiota of Atlantic salmon. *Sci Rep* **6**, 19707. https://doi.org/10.1038/srep19707

Løvoll, M., Wiik-Nielsen, C.R., Tunsjo, H.S., Colquhoun, D., Lunder, T., Sorum, H. and Grove, S. (2009) Atlantic salmon bath challenged with *Moritella viscosa*–pathogen invasion and host response. *Fish Shellfish Immunol* **26**, 877–884. https://doi.org/10.1016/j.fsi.2009.03.019

Lunder, T., Evensen, Ø., Holstad, G. and Håstein, T. (1995) 'Winter ulcer' in the Atlantic salmon *Salmo salar*. Pathological and bacteriological investigations and transmission experiments. *Dis Aquat Organ* **23**(1), 39–49.

Madaro, A., Olsen, R.E., Kristiansen, T.S., Ebbesson, L.O., Nilsen, T.O., Flik, G. and Gorissen, M. (2015) Stress in Atlantic salmon: response to unpredictable chronic stress. *J Exp Biol* **218**(Pt 16), 2538–2550. https://doi.org/10.1242/jeb.120535

Martinez Cruz, P., Ibanez, A.L., Monroy Hermosillo, O.A. and Ramirez Saad, H.C. (2012) Use of probiotics in aquaculture. *ISRN Microbiol* **2012**, 916845. https://doi.org/10.5402/2012/916845

Merrifield, D.L., Dimitroglou, A., Foey, A., Davies, S.J., Baker, R.T.M., Bøgwald, J. and Ringø, E. (2010) The current status and future focus of probiotic and prebiotic applications for salmonids. *Aquaculture* **302**(1–2), 1–18. https://doi.org/10.1016/j.aquaculture.2010.02.007

Mohapatra, S., Chakraborty, T., Kumar, V., DeBoeck, G. and Mohanta, K.N. (2013) Aquaculture and stress management: a review of probiotic intervention. *J Anim Physiol Anim Nutr (Berl)* **97**(3), 405–430. https://doi.org/10.1111/j.1439-0396.2012.01301.x

Moriano-Gutierrez, S., Koch, E.J., Bussan, H., Romano, K., Belcaid, M., Rey, F.E. and McFall-Ngai, M.J. (2019) Critical symbiont signals drive both local and systemic changes in diel and developmental host gene expression. *Proc Natl Acad Sci USA* **116**(16), 7990–7999. https://doi.org/10.1073/pnas.1819897116

Nayak, S.K. (2010) Probiotics and immunity: a fish perspective. *Fish Shellfish Immunol* **29**, 2–14. https://doi.org/10.1016/j.fsi.2010.02.017

Newaj-Fyzul, A. and Austin, B. (2015) Probiotics, immunostimulants, plant products and oral vaccines, and their role as feed supplements in the control of bacterial fish diseases. *J Fish Dis* **38**(11), 937–955. https://doi.org/10.1111/jfd.12313

Norwegian Directorate of Fisheries. https://www.fiskeridir.no/Akvakultur/Tall-og-analyse/Akvakulturstatistikk-tidsserier/Laks-regnbueoerret-og-oerret/Matfiskproduksjon

O'Byrne-Ring, N., Dowling, K., Cotter, D., Whelan, K. and MacEvilly, U. (2003) Changes in mucus cell numbers in the epidermis of the Atlantic salmon at the onset of smoltification. *J Fish Biol* **63**, 1625–1630. https://doi.org/10.1111/j.1095-8649.2003.00273.x

Olsen, A.B., Nilsen, H., Sandlund, N., Mikkelsen, H., Sørum, H. and Colquhoun, D.J. (2011) Tenacibaculum sp. associated with winter ulcers in sea-reared Atlantic salmon *Salmo salar*. *Dis Aquat Organ* **94**(3), 189–199. https://doi.org/10.3354/dao02324

Ringø, E., Salinas, I., Olsen, R.E., Nyhaug, A., Myklebust, R. and Mayhew, T.M. (2007) Histological changes in intestine of Atlantic salmon (*Salmo salar* L.) following in vitro exposure to pathogenic and probiotic bacterial strains. *Cell Tissue Res* **328**, 109–116. https://doi.org/10.1007/s00441-006-0323-0

Romstad, A.B., Reitan, L.J., Midtlyng, P., Gravningen, K. and Evensen, Ø. (2013) Antibody responses correlate with antigen dose and in vivo protection for oil-adjuvanted, experimental furunculosis (*Aeromonas salmonicida* subsp. salmonicida) vaccines in Atlantic salmon (*Salmo salar* L.) and can be used for batch potency testing of vaccines. *Vaccine* **31**(5), 791–796. https://doi.org/10.1016/j.vaccine.2012.11.069

Salinas, I., Myklebust, R., Esteban, M.A., Olsen, R.E., Meseguer, J. and Ringo, E. (2008) In vitro studies of *Lactobacillus delbrueckii* subsp lactis in Atlantic salmon (*Salmo salar* L.) foregut: tissue responses and evidence of protection against *Aeromonas salmonicida* subsp salmonicida epithelial damage. *Vet Microbiol* **128**, 167–177. https://doi.org/10.1016/j.vetmic.2007.10.011

Småge, S.B., Brevik, O.J., Duesund, H., Ottem, K.F., Watanabe, K. and Nylund, A. (2016) *Tenacibaculum finnmarkense* sp. nov., a fish pathogenic bacterium of the family Flavobacteriaceae isolated from Atlantic salmon. *Antonie Van Leeuwenhoek* **109**(2), 273–285. https://doi.org/10.1007/s10482-015-0630-0

Småge, S.B., Frisch, K., Vold, V., Duesund, H., Brevik, Ø.J., Olsen, R.H. and Nylund, A. (2018) Induction of tenacibaculosis in Atlantic salmon smolts using Tenacibaculum finnmarkense and the evaluation of a whole cell inactivated vaccine. *Aquaculture* **495**, 858–864. https://doi.org/10.1016/j.aquaculture.2018.06.063

Takle, H.R., Ytteborg, E., Nielsen, K.V., Karlsen, C.R., Nilsen, H.K., Sveen, L. and Nilsen, A. (2015). Sårproblematikk og hudhelse i laks-og regnbueørrettoppdrett. Nofima.

Thorud, K. and Djupvik, H.O. (1988) Infectious anemia in Atlantic salmon (*Salmo salar*). *Bull Eur Assoc Fish Pathol* **8**, 109–111.

Tort, L. (2011) Stress and immune modulation in fish. *Dev Comp Immunol* **35**(12), 1366–1375. https://doi.org/10.1016/j.dci.2011.07.002

Verschuere, L., Rombaut, G., Sorgeloos, P. and Verstraete, W. (2000) Probiotic bacteria as biological control agents in aquaculture. *Microbiol Mol Biol Rev* **64**(4), 655–671.

Vindas, M.A., Madaro, A., Fraser, T.W., Hoglund, E., Olsen, R.E., Overli, O. and Kristiansen, T.S. (2016) Coping with a changing environment: the effects of early life stress. *R Soc Open Sci* **3**(10), 160382. https://doi.org/10.1098/rsos.160382

III

Aquaculture 518 (2020) 734810

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

Improved health and better survival of farmed lumpfish (*Cyclopterus lumpus*) after a probiotic bath with two probiotic strains of *Aliivibrio*

Øystein Klakegg[a,b,*], Siri Myhren[c], Rosemary Alice Juell[d], Marit Aase[d], Kira Salonius[a], Henning Sørum[b]

[a] Previwo AS, Ullevålsveien 68, 0454 Oslo, Norway
[b] Norwegian University of Life Sciences, Faculty of Veterinary Medicine, Department of Food Safety and Infection Biology, Norway
[c] Tjeldbergodden Rensefisk AS, Buhaugvegen 74, 6699 Kjørsvikbugen, Norway
[d] Lerøy Midt AS avdeling Skorild, 7257 Snillfjord, Norway

ARTICLE INFO

Keywords:
Lumpfish
Probiotic
Health
Welfare
Aliivibrio

ABSTRACT

Cleaner wrasse eat sea lice (*Lepeophtheirus salmonis*) directly from the skin of the Atlantic salmon (*Salmo salar*). To reduce harvest from the wild wrasse populations and to increase the quality and availability of cleaner fish, lumpfish (*Cyclopterus lumpus*) have been increasingly developed as a service industry to the farming of Atlantic salmon in the last few years. Acquired resistance against the de-lousing chemicals is occuring at an increasing speed particularly in mid- and western-Norway has made the farming of lumpfish more important in the control of the sea lice infestations in farmed Atlantic salmon.

Outbreaks of disease in farmed lumpfish occur in all its life stages. Some outbreaks of disease caused by pathogenic bacteria are resulting in high mortality while other bacteria cause lower but more chronic mortality. To reduce the mortality and improve the health of farmed lumpfish microbial enhancement with a probiotic bath approach has been tested.

In five trials at two lumpfish aquaculture facilities in Norway, two probiotic isolates of *Aliivibrio* bacteria were added to the rearing water. The lumpfish were bathed in the probiotic bacteria in 10–30 min, the cfu (colony-forming units) of probiotic bacteria in the rearing water varied from 7.5×10^5 to 5×10^7/mL. The average weights of the lumpfish at time of bathing were from 0.025 g to 16.3 g in the various trials. The lumpfish were observed and sampled from 45 to 87 days after bathing. Growth, ulcers and survival were recorded.

The survival of lumpfish in trials 3, 4 and 5 in which the fish were bathed 1 to 2 weeks before vaccination was significantly better for the probiotic exposed groups compared to the control groups. In trials 4 and 5, a natural outbreak of disease caused by *Moritella viscosa* occurred. The groups exposed to probiotic bacteria had significantly fewer ulcers than the control groups.

The growth of the fish exposed to probiotic bacteria at weight 0.025 g was significantly better in the bathed group compared to the controls. In three trials the growth of the probiotic exposed lumpfish one to two weeks before vaccination was also higher than non-exposed fish.

Bathing of lumpfish with two *Aliivibrio* bacteria increased growth and made the fish more resistant to bacterial diseases. To our knowledge, this is the first scientific report of probiotic bacteria applied to lumpfish.

1. Introduction

The first experiments using cleaner fish for Atlantic salmon to control sea lice infestations in Norway was performed in the late 1980s (Bjordal, 1991). Since then there has been a shift from use of wild caught cleaner fish to mostly farmed cleaner fish. In 2017, more commercial farmed cleaner fish (31.7 million) were used in Norwegian salmon production than wild caught cleaner fish (22.9 million) (Norwegian Directorate of Fisheries: Norwegian Aquaculture statistics). Lumpfish (*Cyclopterus lumpus*) and ballan wrasse (*Labrus bergylta*) are the dominating species of cleaner fish used in Norwegian aquaculture today (Norwegian Directorate of Fisheries: Norwegian Aquaculture Statistics).

Lumpfish are more active and eat sea lice at water temperatures, as low as 4 °C, about 2 °C lower than ballan wrasses (Brooker et al., 2018; Nytrø et al., 2014). Therefore, lumpfish are particularly well suited for delousing Atlantic salmon under Norwegian conditions, in relatively

* Corresponding author at: Previwo AS, Ullevålsveien 68, 0454 Oslo, Norway.
E-mail addresses: oystein.klakegg@previwo.no, post@previwo.no (Ø. Klakegg).

https://doi.org/10.1016/j.aquaculture.2019.734810
Received 23 June 2019; Received in revised form 25 November 2019; Accepted 1 December 2019
Available online 03 December 2019
0044-8486/

cold water (Imsland et al., 2018). All lumpfish (29.7 million in 2017) used as cleaner fish in Norwegian salmon farming today originate from commercial aquaculture lumpfish farms (Norwegian Directorate of Fisheries: Norwegian Aquaculture Statistics).

The welfare for lumpfish in industrial production is often reduced, and the mortality is often high. The exact numbers of loss are unknown, but mortality as high as 100% is suggested (Poppe, 2017). In a survey in five Atlantic salmon net pen sites with 79,000 lumpfish, conducted in 2013 in Norway an average of 48% mortality was reported, varying from 39% to 100%. Of the 13,864 dead lumpfish categorized regarding cause of death in the same survey from 2013 in Norway, 75% were categorized as bacterial infections (Nilsen et al., 2014). Bacterial infections are among the main causes for the high mortality rate seen in lumpfish (Hjeltnes et al., 2017).

Several different bacteria as *Pasteurella* spp, *Vibrio anguillarum, Vibrio ordalii, Aeromonas salmonicida, Pseudomonas anguilliseptica, Moritella viscosa, Tenacibaculum maritimum* and *Piscirickettsia salmonis* are verified to cause disease and mortality to lumpfish (Alarcon et al., 2016; Marcos-Lopez et al., 2013; Marcos-Lopez et al., 2017; Powell et al., 2018; Småge, Frisch, Brevik, Watanabe, & Nylund, 2016). The relevance of other bacterial pathogens frequently identified from lumpfish, such as *Aliivibrio logei, Aliivibrio wodanis, Vibrio splendidus* and *Vibrio tapetis* are uncertain (Gulla et al., 2017; Gulla et al., 2015).

A continued use of lumpfish in aquaculture warrants further efforts to improve fish welfare, which includes methods to monitor and reduce both disease and mortality. Use of probiotic bacteria could be part of the solution. Since the first use of probiotic bacteria to fish in aquaculture bacteria have been applied to several fish species, among others: cod (*Gadus morhua*), turbot (*Scophtalmus maximus*), rainbow trout (*Oncorhynchus mykiss*), European bass (*Dicentrarchus labrax*), European eel (*Anguilla anguilla*) and Atlantic salmon (Balcazar et al., 2006; Hoseinifar et al., 2018; Kozasa, 1986). The use of *Vibrio* spp. (*V.alginolyticus* and *V. fluvialis*) as probiotic bacteria to inhibit pathogens has previously been reported (Austin et al., 1995; Irianto and Austin, 2003). There has been no report of probiotic bacteria reducing mortality caused by *Moritella viscosa* earlier.

Our objective in this study was to investigate if exposing probiotic *Aliivibrio* spp. to lumpfish increased growth and resistance against bacterial diseases and reduced mortality.

2. Materials and methods

2.1. Bacteria

In all trials we used *Aliivibrio* sp. strain B1–25, 18–1/2013 mandib V11, NCIMB 42593 1 and *Aliivibrio* sp. strain B1–24, 18–1/2013 kidn V12, NCIMB 42592 isolated from Atlantic salmon. In all trials the portion of Strain B1–24 and B1–25 was approximately to one to one. Time of bathing with bacteria, concentration of probiotic immersion and water and colony forming units per mL. of water are shown in Table 1.

2.2. Lumpfish facilities

The trials were conducted at two different lumpfish farming facilities in Mid-Norway. Trial 1, 3, 4 and 5 were conducted at farming facility 1, and trial 2 at farming facility 2. Both sites were flow-through farming facilities.

2.2.1. Lumpfish farming facility 1

Lumpfish farming facility 1 was a land-based facility producing lumpfish from egg to fish ready for commercial transfer into Atlantic salmon sea pens. The seawater was pumped from two different water intake pipes at 80 and 100 m depth, respectively. The water passed through a Bernoulli filter with a pore size of 100 μm. The water was then disinfected with ozone (O^3) with a redox value of 500 mV preceding the passage through a UV disinfection unit (wavelength around 254 nm) with a dose of 40 kJ/cm^2. Then the water was saturated with O^2, to 100%, before it was distributed into the different tanks. The average retention time of the water in each tank was approximately 60 min.

2.2.2. Lumpfish farming facility 2

Lumpfish farming facility 2 was a land-based facility producing lumpfish from fry of approximately 0.5 g to fish ready for commercial transfer into Atlantic salmon sea pens. The water at facility 2 was pumped from a fjord at a depth of 30 m. All water passed through a Bernoulli filter with pore size 200 μm before being disinfected by an UV disinfection unit (200–400 nm wavelength) with a minimum dose of 57 mJ/cm^2). Oxygen was added and maintained at 90–110% saturation. The water retention time was 60 to 80 min.

2.3. Lumpfish

The number of lumpfish in each trial, average weights at time of application of probiotic bacteria and tank volumes are shown in Table 1. The lumpfish were not vaccinated before being exposed to probiotic bacteria.

The fish in each trial originated from > 10 females and > 2 males. The probiotic group and control group in trial 1 and 2 hatched the same day, but from different parents. In trial 3, 4 and 5 the probiotic groups and the control groups in the same trial originated from the same parents. The fish at lumpfish farming facility 1 originated from wild fish captured outside the island Averøy, Møre og Romsdal, Norway. The eggs were fertilized and disinfected with buffodine (10 mg/L for 10 min). The fertilized eggs were then transported to lumpfish farming facility 1 where they hatched at approximately 280 to 300 day degrees. The fish at lumpfish farming facility 2 originated from wild fish captured outside Troms in Northern Norway.

The eggs were fertilized and disinfected with buffodine (10 mg/L for 10 min). The fertilized eggs were then transported to Senja akvakultursenter where they hatched around August 1st 2017. November 13th the fry were loaded on a truck and transported to lumpfish farming facility 2 where they arrived the next day.

2.4. Bathing procedures and farming management in the trials

In all trials the intake water was turned off and the O^2 level in the tank was monitored. In trial 1, 3, 4 and 5 the water level was lowered to 1.5 m^3, 6.36 m^3, 4 m^3 and 4 m^3, respectively. In trial 2 the water level was not lowered, hence the fish were bathed in 7.9 m^3 water. After the water volume was reduced, the bacteria were added, by pouring, 15 L, 127 L, 27 L, 40 L and 40 L, respectively. After 10 to 30 min (see Table 1), the water was turned on again gradually filling the tanks to the original level of water. In trial 4 the water was turned on 10 min before planned, due to a drop in O^2-saturation in the water to under 65%, probably due to relatively high concentration of lumpfish in the water, 123 kg/m^3. The concentration of lumpfish in the water in the other trials varied from 2 kg/m^3 (trial 1) to 43 kg/m^3 (trial 5) during bathing with the probiotic bacteria. The fish were observed visually during the bathing procedure. While the fish were bathed and exposed to the bacteria, samples from both the bacterial culture and the water with the exposed fish were collected and analysed for bacterial numbers as described in 2.5.

We performed five individual trials with similar methods but with some differences in the concentration of bacteria. The fish groups and duration of exposure are presented in Table 1.

2.4.1. Trial 1

In trial 1 the lumpfish were transferred from one department of the facility to another the same day as they were exposed to the probiotic bacteria. The fish were fed with dry feed (Gemma wean 0, Skretting) and the amount of feed given to the lumpfish was adjusted to appetite, with some degree of overfeeding.

Table 1
Number of lumpfish, average weights of lumpfish at time of application of probiotic bacteria, tank volume, concentration of the probiotic solution/water, duration of application of probiotic bacteria and CFU per mL and water temperature during trials.

Trial	Number of lumpfish	Average weight Gram (start)	Tank volume (start)	Conc. Probiotic immersion/water	Duration of treatment (until, dilution	CFU *Aliivibrio* spp/ mL water	Water temperature during trial (°C)
1. Lumpfish farming site 1	120,000	0.025	2 m^3	1/100	20 min	5×10^7	9 °C
Aug 2017- Sept 2017	120,000 (C)*	0.025	2 m^3				9 °C
2.Lumpfish farming site 2	34,000	1.09	7.9 m^3	1/62	30 min	$7{,}5 \times 10^5$	6.3–10 °C (7.5 °C average)
Dec 2017-March 2018	38,000(C)	0.91	7.9 m^3				6.3–10 °C (7.5 °C average)
3. Lumpfish farming site 1	5700	7.2	9 m^3	1/235	30 min	5×10^7	7,6 °C-
June 2018- Aug 2018	13,000(C)	7.6	9 m^3				7,6 °C
4. Lumpfish farming site 1	30,200	16.3	9 m^3	1/100	10 min	2×10^7	9 °C
Oct 2018- Dec 2018	24,600(C)	13.3	9 m^3				9 °C
5. Lumpfish farming site 1	11,700	14.6	9 m^3	1/100	10 min	2×10^7	9 °C
Oct 2018- Dec 2018	15,300(C)	14.9	9 m^3				9 °C

(C)* Controls.

2.4.2. Trial 2

The fish were exposed to probiotic bacteria 29 days after arriving at the lumpfish farming facility 2. The fish were kept in two types of tanks. First, they were in circular tanks with a diameter of 3 m and a volume of 7.9 m^3. As the fish grew, they were transferred to larger, 9 m^3, tanks (VK tank) after sorting. The fish were fed 0.8 mm pelleted feed (Clean assist, Skretting) and the amount of feed was adjusted to appetite, with some degree of overfeeding. After being transferred to VK tanks they were fed with 1.0 mm pelleted feed (Clean assist, Skretting).

Some of the fish ended up being mixed (control and treated) during transfer, hence only the smallest group where followed for the last month.

2.4.3. Trial 3

The lumpfish were pumped from the tanks in which they had been exposed to the probiotic bacteria with an impeller pump and manually vaccinated against *Vibrio anguillarum, Aeromonas salmonicida* subsp. *achromogenes, Pasteurella piscicida* (Alpha marine micro 3.1, Pharmaq AS) 11 days after exposure to the probiotic bacteria. After vaccination the fish were transported by gravity to equal clean tanks. After this the fish were not moved again before delivery for sale. The fish were fed with dry pelleted feed 1.5 mm and 1.8 mm (Gemma Diamond 1.5 and Gemma Diamond 1.8, Skretting) and the amount of feed was adjusted to appetite, with some degree of overfeeding.

2.4.4. Trial 4 and 5

The lumpfish were pumped from the tanks in which they were exposed to the probiotic bacteria by the use of an impeller pump and manually vaccinated with Alpha marine micro 3.1 (Pharmaq AS) seven days after exposing the lumpfish with the probiotic bacteria and then transported by gravity to equal clean tanks. In this trial (the 18–1 generation) both the probiotic exposed and the control group, were split into two tanks each after vaccination. The fish were fed with pelleted feed of 1.5 mm and 1.8 mm (Clean Assist 1.5 and Clean Assist 1.8, Skretting) and the amount of feed was adjusted to appetite, with some degree of overfeeding.

Due to an outbreak of disease caused by *M. viscosa*, the fish in both trial 4 and 5 were treated with florfenicol in medicated feed, (Floraqpharma vet. medicated pellets 2 g/kg, Skretting). The duration of the antibiotic treatment was 10 days and the feeding intensity were 2% of the biomass per day. The amount of feed increased to 4% of the biomass per day during the antibiotic treatment. The treatment started, November 15th 2018, 31 days after the exposure to the probiotic bacteria in all tanks except one tank of probiotic exposed lumpfish (18–1 generation, tank 438) and one tank with control lumpfish 18–1 generation, tank 427). In these two tanks, the antibiotic treatment started 38 days after application of the probiotic bacteria. The control fish in trial 5 (17–16 generation) were also treated with florfenicol for a second time, because the mortality rate was still high after the first treatment.

2.5. Cultivating of probiotic bacteria

The probiotic bacterial strains were cultivated from frozen stock consisting of bacterial cells stored in broth (Luria-Bertani broth with 2.5% NaCl and glycerol 20%) at −80 °C. The frozen cells were first incubated on blood agar plate (OXOID, CM0271, blood agar base nr.2, with 5% bovine blood and 2.5% (*w/v*) NaCl at 12 °C for two to four days. From the blood agar plates, the bacteria were inoculated and cultivated further in liquid broth (Luria-Bertani broth with 2.5% NaCl) at 12 °C for two to four days with shaking at 220 rpm, first in a small volume, and then successively in larger volumes. The two probiotic bacterial strains were cultivated in monocultures that were mixed before the last step of the cultivation procedure and distributed to the rearing water as a two-culture-broth.

When the fish were exposed to the probiotic bacteria samples were taken from the bacterial culture and diluted ten-fold in phosphate buffered saline (2.5%) PBS broth and 100 µl from three different dilutions were spread with an L-spreader on a blood agar plate at 12 °C before the colony forming units (cfu) were counted when visible colonies could be observed, usually after two days. The figures of cfu of *Aliivibrio* spp. in trial 1, 3, 4 and 5 varied from 2×10^9 to 1.2×10^{10} per mL in the probiotic immersion. In trial 2 the cfu was 5×10^7 per mL in the immersion solution and 7.5×10^5 cfu per mL in the water used for bathing the fish. These bacterial concentrations were from 26 to 240 times less cfu of *Aliivibrio* spp. per mL compared to the other trials. Cultivation from the bacterial culture when bathing the lumpfish in trial 2, revealed that the bacterial culture was not pure and was contaminated with *Micrococcus* sp.

2.6. Assessment of ulcers (trial 4 and 5), mortality, growth and feed consumption

The mortality in trials 1, 2 and 3 were reported daily. In trial 4 and 5 the total numbers of dead and discarded lumpfish, mostly due to ulcers, were reported daily. In trial 4 and 5 external ulcers to tail and body were also assessed. The scoring was categorized as: a) no ulcers, b) small ulcers < 5 mm and superficial, not penetrating dermis, and c) larger ulcers > 5 mm or penetrating the dermis.

2.7. Sampling, cultivation and identification of bacteria from ulcers and kidney

Specimens from the ulcers and head kidney of moribund fish with large lesions were sampled with an inoculation loop and cultured directly on Marine Agar (Difco™ Marine Broth 2216,) and blood agar

(OXOID CM0271, Blood Agar Base No.2, with 2,5% NaCl) and incubated at 12 °C for 48 to 96 h. To identify the species of the bacteria we used 16S *rRNA* gene analysis. Universal primers (27F and 1492R) and the GoTaq® DNA polymerase (Promega) kit was used to amplify the 16S rRNA gene following the protocol described by the manufacturer. The PCR products were checked for the expected product size after separation in agarose gel electrophoresis. The purified PCR products were sequenced using sequencing service (GATC, now Eurofins Scientific, Konstanz, Germany).

Identification of the bacteria was done by blasting the 16S *rRNA* gene sequences using Basic Local Alignment Search Tool (BLAST®) at the NCBI website which compared the nucleotide sequences to the sequence database.

2.8. Statistics

Calculations of mean, standard deviation of the mean, standard error of the mean, upper and lower 95% confidence interval of the mean and probabilities based on Fisher's exact test, were all conducted in JMP® (JMP®, version (Pro 14). SAS institute, Cary, NC, 1989–2019).

3. Results

3.1. Mortality

In trial 4 and 5 all six tanks were hit by disease and mortality caused by *M. viscosa* a few days after exposure to the probiotic (Figs. 1 and 2). This was the main cause of mortality in these trials. Four of the tanks were given antibiotic, florfenicol, from 3.5 weeks after exposure to the probiotic bacteria, while the remaining two tanks were given florfenicol from 4.5 weeks after exposure. The mortality and morbidity were reduced after the antibiotic treatment. The probiotic exposed groups of lumpfish had significant lower weekly mortality in trial 4 and 5 from one week after the probiotic treatment to two weeks after treatment with antibiotic in medicated feed in all trials and tanks (Table 2 and Figs. 3 and 4).

The total mortality (discarded and mortality in trial 4 and 5) varied from 4.47% to 33.3% in the probiotic exposed groups and from 6.16% to 32.12% in the controls (Table 2). In trial 1, 2, 3 and 5 the probiotic exposed groups had a higher mortality than the control groups the first two days after exposure. From one week after application of the probiotic bacteria to the end of the trials, the bathed groups had lower mortality and discards than the controls groups in all trials (Table 2).

In trial 3 the weekly mortality from week three after the probiotic exposure to the end of the trial was significant lower in the exposed group ($p < 0.05$) (Fig. 5). In trial 4 and 5 the weekly mortality from week two after the probiotic exposure to two weeks after treatment with florfenicol, mortality and discarding were from 29% to 78% lower in the exposed group ($p < 0.02$). In trial 2 both growth and survival were higher in the control group. During the first two weeks after bathing the control group mortality was 0.5% lower than the probiotic enhanced group. However, from two weeks after application of the probiotic bacteria to the end of the trial the mortality in the probiotic treated group was 0.16 percent point lower than the mortality in the control group. In trial 1, the total mortality was highest in the probiotic applicated group. This may be due to stress as the fish were transported, on the same day as the probiotic application was performed, from one department within the site to another department. It was the first two days afterbathing that the mortality in the probiotic applicated groups were highest. However, from two days after application of the probiotic bacteria until end of trial the mortality in the probiotic bathed group was significantly lower than in the control group ($p < 0.01$), Fisher's exact test).

Fig. 1. Lumpfish with a large ulcer caused by *M. viscosa*.

Fig. 2. Closer view of an ulcer caused by *M. viscosa* on the side, penetrating the skin.

3.2. Growth

In trial 1, the probiotic bathed group had a significantly higher growth ($p < 0.01$) and mean weight was 33.3% larger than the mean weight in the control group at the end of trial (Table 3). In trial 2, at lumpfish farming facility 2, the growth in the control group was slightly better than in the treated group (Table 3). In trial 3, the probiotic exposed group was 28% larger at end of trial despite they were slightly smaller at start of the trial (Table 3).

In trial 4, the control group had overall the highest growth (Table 3).

In trial 5, the probiotic exposed group grew significantly ($p < 0.05$) better from exposure until 48 days after exposure (Table 3). Then, the control group grew best from day 48 after exposure until day 68 after probiotic exposure. The group was treated with antibiotic 31 days after probiotic exposure, and the viability of the probiotic bacteria in the fish was most likely reduced by the antibiotic treatment.

3.3. Ulcer

In trial 4 and 5, there were outbreak of bacterial infection caused by *M. viscosa* resulting in ulcers and an increased mortality in all tanks. The ulcers varied in size, the largest were 25–30 mm in width, but most often between 3 and 10 mm. The ulcers penetrated the skin, and in some cases extended into the muscle tissue (Figs. 1 and 2).

During the disease outbreak there were significantly ($p < 0.05$) fewer lumpfish with ulcers in the probiotic exposed groups compared to the control groups (Table 4). After treatment with florfenicol in all groups the difference in ulcer frequency between probiotic exposed and control groups decreased.

From all the ulcers and 60% of the kidneys we cultivated β-hemolytic colonies resembling *M. viscosa.* The results from the BLAST® using 16S rDNA sequences from the isolated β-hemolytic isolates matched with *M. viscosa*. From nearly 50% of the ulcers vibrio-like colonies were also growing in addition to *M. viscosa*, but more sparse. The results from the BLAST® using 16S rDNA sequences matched with *V. splendidus*.

4. Discussion

This study demonstrates that lumpfish are more robust to disease caused by *M. viscosa*, after enhancing the bacterial microbiota with probiotic bacteria. Both the mortality ($p < 0.01$) and the incidence of

Table 2
Cumulative mortality (and discarding in trial 4 and 5) in all trials, two days after application of the probiotic bacteria, one week after application of the probiotic bacteria, from one week after application of the probiotic bacteria to end of trials and total.

	Cumulative mortality and discarding (trial 4 and 5) in percent									
	First 2 days		First 7 days		Total			From 7 days to end		
Trial	Bacteria	Control	Bacteria	Control	Bacteria	Control	p	Bacteria	Control	p
1	10.21	3.67	13.94	6.99	33.30	29.88	> 0.99	19.36	22.89	< 0.01
2	0.256	0.14	1.43	0.91	6.52	6.16	0.98	5,09	5,25	0.17
3	0.07	0.05	0.61	0.46	5.63	7.24	< 0.01	5,02	6,78	< 0.01
4	0.34	1.22	0.93	2.56	4.47	9.86	< 0.01	3,54	7,30	< 0.01
5	0.39	0.17	2.38	1.71	16.47	32.12	< 0.01	14.09	30.41	< 0.01

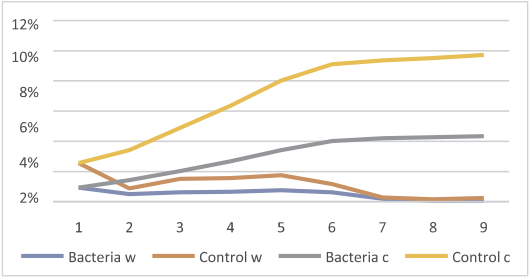

Fig. 3. Mortality and discarding, weekly (w) and cumulative (c), the first 9 weeks after application of probiotic bacteria in trial 4. Treatment with florfenicol started 4.5 (one bacteria tank and one control tank) and 5.5 (the second bacteria tank and the second control tank) weeks after bathing with probiotic bacteria.

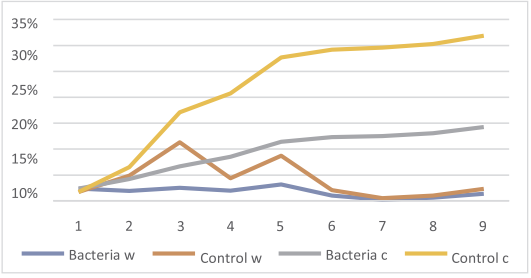

Fig. 4. Mortality and discarding, weekly (w) and cumulative (c), the first 9 weeks after application of probiotic bacteria in trial 5. Treatment with florfenicol started 4.5 weeks after bathing with probiotic bacteria.

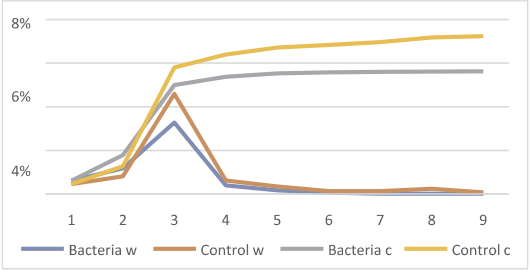

Fig. 5. Mortality and discarding, weekly (w) and cumulative (c), the first 9 weeks after application of probiotic bacteria in trial 3.

ulcers ($p < 0.05$) were significant lower in the bacteria enhanced groups, compared to the controls in the two trials hit by a natural *M. viscosa* outbreak. The probiotic *Aliivibrio* spp. delivered here appear to make the lumpfish more robust against this disease and may contribute to improvements with regard to other diseases and problems. The growth of the lumpfish from bathing with probiotic bacteria to end of trials, was significant larger in the bacteria enhanced groups in trial 1, 3 and 5. In trial 4 the bacterial enhanced group was significant larger ($p < 0.05$) than the control group at end of the trial. But the bacterial enhanced group was also significant largest at start of the trial ($p < 0.01$). Overall the control group had the largest growth from time of bacterial enhancement to end of trial in trial 4. In trial 2 the overall mortality was lower in the control group and the growth was largest in the control group. The reason that the probiotic applicated group in trial 2 did not perform better regarding mortality or growth than the control, might be because the concentration of the probiotic bacteria was too low. In trial 2 the concentration was 7.5×10^5 cfu/mL, compared to the other trials that all were above 2×10^7 cfu/mL. Dosage level of bacteria is important both to get probiotic effect and in challenge trials with pathogens (Merrifield et al., 2010). The result in trial 2, as in trial 1, could also be affected that the lumpfish in the control groups and the lumpfish in the treated groups originated from different parents. The probiotic stock culture in trial 2 was also contaminated with *Micrococcus* sp. so the lumpfish in trial 2 was enhanced in both *Aliivibrio* spp. and *Micrococcus* sp. We do not know any negative impact of *Micrococcus* sp. to lumpfish, but it is probably not positive that the culture scheduled to be only *Aliivibrio* spp., also contained *Micrococcus* sp.

In the trials hit by *M. viscosa* infection both mortality and incidence of ulcers in the bacterial enhanced groups and controls became more equal after treatment with florfenicol. This was as expected as the florfenicol also combatted the probiotic *Aliivibrio* spp., and hence diminished the effect of the probiotic bath after treatment with florfenicol. Very little has been published regarding the microbiome of lumpfish, but our research (not yet published) indicates that the microbiota of Atlantic salmon and lumpfish are overlapping. Many of the same bacteria that cause problems for lumpfish also cause problems for Atlantic salmon; *V. anguillarum, A. salmonicida, M. viscosa*, and *T. maritimum* among others (Alarcon et al., 2016; Einarsdottir et al., 2018; Gulla et al., 2015; Marcos-Lopez et al., 2013; Marcos-Lopez et al., 2017; Småge et al., 2016).

It is not only the pathogenic bacteria which are common between the species. Cultivated bacteria from healthy salmon are also found in lumpfish, which support that farmed lumpfish share some bacterial commensals as well. The probiotic *Aliivibrio* spp. used in this study were first identified from Atlantic salmon. Some of the bacteria that were found in healthy untreated lumpfish are the same probiotic *Aliivibrio* spp. found on Atlantic salmon (data not published). Since the microbiota of animals are related to their surroundings, it is reason to believe that the same *Aliivibrio* spp. also can be found in other species and maybe act as a probiotic also to other species.

Fish skin is not the solid barrier against bacteria as has traditionally been thought. Bacteria can pass through intact Atlantic salmon skin and

Table 3
Growth from start of trials to end of trials.[1] In trial 4 and 5, end of trial was set to the weighting Nov. 27th, due to treatment of florfenicol. [2] In trial 2 only a fraction of the fish, the smallest of the smallest, were followed to the end of the trial. [3] Start weights in trial 1 and end weights in trial 3 was not done by individual weighting of fish, but gross weight of many fish, 200 control and 200 bacteria enhanced lumpfish in trial 1 and 57 control and 57 bacteria enhanced lumpfish in trial 3.

Trial		Weight start mean (g)	Lower 95% Mean	Upper 95% Mean	Weight end mean (g)	Lower 95% Mean	Upper 95% Mean	Growth start to end (%)
1	Control	0.025	na[3]	na	0.150	0.138	0.163	600
	Bacteria	0.025	na	na	0.200	0.187	0.213	800
2	Control	0.91	0.842	0.987	5.12[2]	4.70	5.53	563
	Bacteria	1.09	1.02	1.15	5.14[2]	4.74	5.53	472
3	Control	7.55	7.28	7.81	16.5	na[3]	na	219
	Bacteria	7.24	6.97	7.50	21.2	na	na	293
4	Control	13.31	12.71	13.90	39.1[1]	37.9	40.4	294
	Bacteria	16.32	15.54	17.10	41.8[1]	40.4	43.2	256
5	Control	14.87	14.05	15.69	46.9[1]	45.0	48.9	315
	Bacteria	14.56	14.03	15.09	50.6[1]	48.1	53.1	330

Table 4
Ulcer in trial 4 and 5 at different dates of scoring. n = number of lumpfish scored, p: Fisher's exact test t, *pooled measurements Nov. 7th and Nov 27th.

Date	Bacteria			Control			p	
	n	Ulcer	No ulcer	n	Ulcer	No ulcer		
Trial 4 Generation 18–1								
Oct. 15th	110	0	110	110	0	110		
Nov. 7th	204	4	200	204	11	193	0.067	0.014*
Nov. 27th	204	20	184	204	31	173	0.056	
Dec. 17th	102	6	96	204	16	188		
Trial 5 Generation 17–16								
Oct. 15th	110	0	110	110	0	110		
Nov. 7th	102	1	101	102	7	95	0.033	0.004*
Nov. 27th	102	17	85	102	30	72	0.028	
Dec. 17th	102	8	94	102	9	93		

can be detected in the blood a few minutes after exposure even when the bacteria are not exposed to the gills, mouth or gut (Kashulin and Sørum, 2014). The skin may be the major and most important route of entry for many other bacteria into the fish via the water. The surrounding water is important for the gut microbial colonization of the fish larvae in several fish species (Egerton et al., 2018). It is likely that most fish species have communication with the bacteria in the surrounding water, and it is not unlikely that the lumpfish passively or actively will maintain, increase or change their bacterial microbiota. In other species it has been revealed that the vertebrate hosts often make efforts to keep the right species of bacteria (Ezenwa et al., 2012). This encourages the idea that purposeful microbial intervention may have a long-term and functional effect in this species.

Adding probiotic bacteria to the rearing water simplifies the application mode for probiotic bacteria, compared to administration with the feed and has been done for more than 25 years (Austin et al., 1995). There is for example no concern regarding loss of viability due to the low bactericide pH in the stomach or high processing temperature in feed manufacturing.

Aquaculture alters the living environment for the fish and its bacterial microbiome to a great extent (Ramirez and Romero, 2017).

Rearing temperature, density of fish, feeding, feces and RAS technology, among other things, all have great impact on the diversity and abundance of bacteria surrounding and colonizing the fish. Presence of feed and feces in the rearing water results in an increase in dissolved organic matter (DOM) and disinfection of the intake water may tend to result in selection of bacterial species that are opportunistic and have high growth rates. Water disinfection as UV-disinfection of the intake water is not sufficient to kill all bacteria and can also alter the relative quantum of the different bacterial species in the water (de Carvalho, 2017; Moreno-Andres et al., 2018). Altogether this contributes to the phenomenon that bacteria in nature and in artificial rearing systems are often different, regarding amount, relative amount and diversity (Banerjee and Ray, 2017; Lee et al., 2016). The artificial rearing system often produces systems with low bacterial diversity, low bacterial control and poor stability for perturbations and disturbing of the delicate symbiosis of fish and bacteria, and dysbiosis may occur (Brugman et al., 2018; Vadstein et al., 2018).

In a survey in Chile of the marine yellowtail kingfish (*Seriola lalandi*) they found that the microbiome of wild fish and fish from a recirculating aquaculture system (RAS) was significantly different (Ramirez and Romero, 2017). The predominant phylum of wild yellowtail was *Proteobacteria* and the class *Gammaprotebacteria*, such as *Psychrobacter, Pseudomonas* and *Acinetobacter*, while the dominant phylum from yellowtail from aquaculture was *Firmicutes*, such as *Staphylococcus, Clostridium, Aerococcus* and *Brevibacterium* (Ramirez and Romero, 2017). Several of the *Gammaprotebacteria* found in abundance in wild yellowfish has been described as beneficial probiotic bacteria (Ramirez and Romero, 2017). *Aliivibrio* spp. are also in the class *Gammaprotebacteria.*

We know that the microbiota is important for many biological processes in the body (Brugman et al., 2018; Falcinelli et al., 2015; Lescak and Milligan-Myhre, 2017). Since aquacultured species live in different bacterial environments compared to the wild fish, optimizing the bacterial surrounding should be a priority. Adding natural probiotic bacteria making the fish more robust, could prevent some of the fish from developing illness and finally death. This will help making the aquaculture more sustainable.

How the *Aliivibrio* spp. strains help the fish to resist diseases better and probably grow faster, is still unknown. However, it is proposed that probiotic bacteria act both by inhibiting, outcompeting and displacing other potentially malevolent bacteria as well as directly helping the fish to become more robust and utilize the food better. It is known that bacteria use many methods, such as quorum-sensing, to communicate with other bacteria and with the host (Bassler and Losick, 2006; Camilli and Bassler, 2006; Pacheco and Sperandio, 2009).

It is reported that bacteria can cause upregulation of genes responsible for physiological processes such as metabolism, pathogen protection and maturation of the immune system (Esser et al., 2018). These modes of action may contribute to the beneficial effects seen with *Aliivibrio* spp. Many bacteria have inhibiting effects on other bacteria and some probably have also beneficial effects (Balcazar et al., 2006; Dimitroglou et al., 2011; Verschuere et al., 2000).

M. viscosa and *A. wodanis* both often co-exist in ulcers and internal organs in Atlantic salmon. It is known that that the presence of *A. wodanis* has an inhibiting effect on *M. viscosa* by altering the gene expression of *M. viscosa*, maybe as a result of inhibiting bacteriocin activity (Hjerde et al., 2015; Karlsen et al., 2014). *Aliivibrio wodanis* and the two *Aliivibrio* spp. used in these trials are phylogenetic relatively close. The inhibiting mechanisms of the two *Aliivibrio* spp. against *M. viscosa* in this trial could be the same as *A. wodanis*` inhibiting mechanisms of *M. viscosa*, without *A. wodanis*` bad effects of the fish.

The reason that two *Aliivibrio* strains were used together, and not one by one, is that bacteria often help each other and communicate

with each other and overlap and amplify each other regarding host effects (Kinnula et al., 2017).

In conclusion we have shown that bathing lumpfish with two probiotic strains of *Aliivibrio* species has made the fish more robust and more resistant against bacterial disease caused by *M. viscosa*. Both mortality and incidence of ulcers were significant lower in the bacterial enhanced groups compared to the controls. Regarding growth, the trials did not reveal an equally clear picture, but in three of the four trials were the bacterial enhanced groups were bathed in a bacterial concentration of $> 1 \times 10^7$ CFU/mL of water, the growth was significantly better in the bacterial enhanced groups.

It is difficult to evaluate the continued effect after launch of the cleaner lumpfish into the open net pens in the sea, but the results within the rearing facilities could be a good model for challenges in open sea, so higher survival, productivity and welfare for lumpfish in open salmon cages may result from probiotic application in the land facilities. Bathing lumpfish is probably best before stressful operations or before periods of increased infection pressure or susceptibility for infections, as vaccination or transferring to sea cages.

The interactions of bacteria and their effects of the host is an area of research that probably will be widely explored in the future, and it not unlikely that we in the future will have probiotics consisting of many different bacteria for aquaculture systems, and that their relative contribution will result in specific and targeted probiotic enhancement.

Funding

The Research Council of Norway (260204/O30), and Previwo AS.

Declaration of Competing Interest

Kira Salonius is Managing Director, Øystein Klakegg is employee and Henning Sørum is one of the owners and founder of Previwo AS, the company that develop the bacterial probiotics we have used in these trials.

Acknowledgment

Thanks to Klemet Steen, Jan Frode Snekvik, Odd Arne Kjørsvik and Svein Martinsen that provide access to the lumpfish sites.

References

Alarcon, M., Gulla, S., Rosaeg, M.V., Ronneseth, A., Wergeland, H., Poppe, T.T., Colquhoun, D.J., 2016. Pasteurellosis in lumpsucker *Cyclopterus lumpus*, farmed in Norway. J. Fish Dis. 39 (4), 489–495. https://doi.org/10.1111/jfd.12366.

Austin, B., Stuckey, L.F., Robertson, P.A.W., Effendi, I., Griffith, D.R.W., 1995. A probiotic strain of *Vibrio alginolyticus* effective in reducing diseases caused by *Aeromonas salmonicida, Vibrio anguillarum* and *Vibrio ordalii*. J. Fish Dis. 18 (1), 93–96. https://doi.org/10.1111/j.1365-2761.1995.tb01271.x.

Balcazar, J.L., de Blas, I., Ruiz-Zarzuela, I., Cunningham, D., Vendrell, D., Muzquiz, J.L., 2006. The role of probiotics in aquaculture. Vet. Microbiol. 114 (3–4), 173–186. https://doi.org/10.1016/j.vetmic.2006.01.009.

Banerjee, G., Ray, A.K., 2017. The advancement of probiotics research and its application in fish farming industries. Res. Vet. Sci. 115, 66–77. https://doi.org/10.1016/j.rvsc.2017.01.016.

Bassler, B.L., Losick, R., 2006. Bacterially speaking. Cell 125 (2), 237–246. https://doi.org/10.1016/j.cell.2006.04.001.

Bjordal, A., 1991. Wrasse as Cleaner-Fish for Farmed Salmon. vol. 16.

Brooker, A.J., Papadopoulou, A., Gutierrez, C., Rey, S., Davie, A., Migaud, H., 2018. Sustainable production and use of cleaner fish for the biological control of sea lice: recent advances and current challenges. Vet. Rec. 183 (12), 383. https://doi.org/10.1136/vr.104966.

Brugman, S., Ikeda-Ohtsubo, W., Braber, S., Folkerts, G., Pieterse, C.M.J., Bakker, P., 2018. A comparative review on microbiota manipulation: lessons from fish, plants, livestock, and human research. Front. Nutr 5, 80. https://doi.org/10.3389/fnut.2018.00080.

Camilli, A., Bassler, B.L., 2006. Bacterial small-molecule signaling pathways. Science 311 (5764), 1113–1116. https://doi.org/10.1126/science.1121357.

de Carvalho, C., 2017. Biofilms: microbial strategies for surviving UV exposure. Adv. Exp. Med. Biol. 996, 233–239. https://doi.org/10.1007/978-3-319-56017-5_19.

Dimitroglou, A., Merrifield, D.L., Carnevali, O., Picchietti, S., Avella, M., Daniels, C., Davies, S.J., 2011. Microbial manipulations to improve fish health and production–a Mediterranean perspective. Fish Shellfish Immunol. 30 (1), 1–16. https://doi.org/10.1016/j.fsi.2010.08.009.

Egerton, S., Culloty, S., Whooley, J., Stanton, C., Ross, R.P., 2018. The gut microbiota of marine fish. Front. Microbiol. 9, 873. https://doi.org/10.3389/fmicb.2018.00873.

Einarsdottir, T., Sigurdardottir, H., Bjornsdottir, T.S., Einarsdottir, E., 2018. *Moritella viscosa* in lumpfish (*Cyclopterus lumpus*) and Atlantic salmon *(Salmo salar*). J. Fish Dis. https://doi.org/10.1111/jfd.12884.

Esser, D., Lange, J., Marinos, G., Sieber, M., Best, L., Prasse, D., Sommer, F., 2018. Functions of the microbiota for the physiology of animal Metaorganisms. J. Innate Immun. 1–12. https://doi.org/10.1159/000495115.

Ezenwa, V.O., Gerardo, N.M., Inouye, D.W., Medina, M., Xavier, J.B., 2012. Microbiology. Animal behavior and the microbiome. Science 338 (6104), 198–199. https://doi.org/10.1126/science.1227412.

Falcinelli, S., Picchietti, S., Rodiles, A., Cossignani, L., Merrifield, D.L., Taddei, A.R., Carnevali, O., 2015. *Lactobacillus rhamnosus* lowers zebrafish lipid content by changing gut microbiota and host transcription of genes involved in lipid metabolism. Sci. Rep. 5, 9336. https://doi.org/10.1038/srep09336.

Gulla, S., Sørum, H., Vågnes, O., Colquhoun, D.J., 2015. Phylogenetic analysis and serotyping of *Vibrio splendidus*-related bacteria isolated from salmon farm cleaner fish. Dis. Aquat. Org. 117 (2), 121–131. https://doi.org/10.3354/dao02938.

Gulla, S., Rønneseth, A., Sørum, H., Vågnes, O., Balboa, S., Romalde, J.L., Colquhoun, D.J., 2017. *Vibrio tapetis* from wrasse used for ectoparasite bio-control in salmon farming: phylogenetic analysis and serotyping. Dis. Aquat. Org. 125 (3), 189–197. https://doi.org/10.3354/dao03140.

Hjeltnes, B., Bornø, G., Jansen, M.D., Haukaas, A., Walde, C., 2017. Fiskehelserapporten 2016 Veterinærinstituttet.

Hjerde, E., Karlsen, C., Sørum, H., Parkhill, J., Willassen, N.P., Thomson, N.R., 2015. Co-cultivation and transcriptome sequencing of two co-existing fish pathogens *Moritella viscosa* and *Aliivibrio wodanis*. BMC Genomics 16, 447. https://doi.org/10.1186/s12864-015-1669-z.

Hoseinifar, S.H., Sun, Y.Z., Wang, A., Zhou, Z., 2018. Probiotics as means of diseases control in aquaculture, a review of current knowledge and future perspectives. Front. Microbiol. 9, 2429. https://doi.org/10.3389/fmicb.2018.02429.

Irianto, A., Austin, B., 2003. Use of dead probiotic cells to control furunculosis in rainbow trout, *Oncorhynchus mykiss* (Walbaum). J. Fish Dis. 26 (1), 59–62.

Karlsen, C., Vanberg, C., Mikkelsen, H., Sorum, H., 2014. Co-infection of Atlantic salmon (*Salmo salar*), by *Moritella viscosa* and *Aliivibrio wodanis*, development of disease and host colonization. Vet. Microbiol. 171 (1–2), 112–121. https://doi.org/10.1016/j.vetmic.2014.03.011.

Kashulin, A., Sørum, H., 2014. A novel in vivo model for rapid evaluation of *Aliivibrio salmonicida* infectivity in Atlantic salmon. Aquaculture 420, 112–118. https://doi.org/10.1016/j.aquaculture.2013.10.025.

Kinnula, H., Mappes, J., Sundberg, L.R., 2017. Coinfection outcome in an opportunistic pathogen depends on the inter-strain interactions. BMC Evol. Biol. 17 (1), 77. https://doi.org/10.1186/s12862-017-0922-2.

Kozasa, M., 1986. *Toyocerin* (*Bacillus toyoi*) as Growth Promotor for Animal Feeding. vol. 4.

Lee, D.E., Lee, J., Kim, Y.M., Myeong, J.I., Kim, K.H., 2016. Uncultured bacterial diversity in a seawater recirculating aquaculture system revealed by 16S rRNA gene amplicon sequencing. J. Microbiol. 54 (4), 296–304. https://doi.org/10.1007/s12275-016-5571-4.

Lescak, E.A., Milligan-Myhre, K.C., 2017. Teleosts as model organisms to understand host-microbe interactions. J. Bacteriol. 199 (15). https://doi.org/10.1128/jb.00868-16.

Marcos-Lopez, M., Donald, K., Stagg, H., McCarthy, U., 2013. Clinical *Vibrio anguillarum* infection in lumpsucker *Cyclopterus lumpus* in Scotland. Vet. Rec. 173 (13), 319. https://doi.org/10.1136/vr.101763.

Marcos-Lopez, M., Ruane, N.M., Scholz, F., Bolton-Warberg, M., Mitchell, S.O., Murphy O'Sullivan, S., Rodger, H.D., 2017. *Piscirickettsia salmonis* infection in cultured lumpfish (*Cyclopterus lumpus L.*). J. Fish Dis. 40 (11), 1625–1634. https://doi.org/10.1111/jfd.12630.

Merrifield, D.L., Dimitroglou, A., Foey, A., Davies, S.J., Baker, R.T.M., Bøgwald, J., Ringø, E., 2010. The current status and future focus of probiotic and prebiotic applications for salmonids. Aquaculture 302 (1–2), 1–18. https://doi.org/10.1016/j.aquaculture.2010.02.007.

Moreno-Andres, J., Acevedo-Merino, A., Nebot, E., 2018. Study of marine bacteria inactivation by photochemical processes: disinfection kinetics and growth modeling after treatment. Environ. Sci. Pollut. Res. Int. 25 (28), 27693–27703. https://doi.org/10.1007/s11356-017-1185-6.

Nilsen, A., Viljugren, H., Røsæg, M.V., Colquhon, D., 2014. Rensefiskhelse-kartlegging av dødelighetssårsaker. Veterinærinstituttets rapportserie 12. .

Nytrø, A.V., Vikingstad, E., Foss, A., Hangstad, T.A., Reynolds, P., Eliassen, G., Imsland, A.K., 2014. The effect of temperature and fish size on growth of juvenile lumpfish (*Cyclopterus lumpus L.*). Aquaculture 434, 296–302. https://doi.org/10.1016/j.aquaculture.2014.07.028.

Pacheco, A.R., Sperandio, V., 2009. Inter-kingdom signaling: chemical language between bacteria and host. Curr. Opin. Microbiol. 12 (2), 192–198. https://doi.org/10.1016/j.mib.2009.01.006.

Poppe, T., 2017. Norsk veterinærtidsskrift nr. 4. pp. s214–s218.

Powell, A., Treasurer, J.W., Pooley, C.L., Keay, A.J., Lloyd, R., Imsland, A.K., de Leaniz, C.G., 2018. Use of lumpfish for sea-lice control in salmon farming: challenges and

opportunities. Rev. Aquac. 10 (3), 683–702. https://doi.org/10.1111/raq.12194.
Ramirez, C., Romero, J., 2017. The microbiome of *Seriola lalandi* of wild and aquaculture origin reveals differences in composition and potential function. Front. Microbiol. 8, 1844. https://doi.org/10.3389/fmicb.2017.01844.
Småge, S.B., Frisch, K., Brevik, O.J., Watanabe, K., Nylund, A., 2016. First isolation, identification and characterisation of *Tenacibaculum maritimumin* Norway, isolated fromdiseased farmed sea lice cleaner fish *Cyclopterus lumpus L.* Aquaculture 464, 178–184. https://doi.org/10.1016/j.aquaculture.2016.06.030.
Vadstein, O., Attramadal, K.J.K., Bakke, I., Olsen, Y., 2018. K-selection as microbial community management strategy: a method for improved viability of larvae in aquaculture. Front. Microbiol. 9, 2730. https://doi.org/10.3389/fmicb.2018.02730.
Verschuere, L., Rombaut, G., Sorgeloos, P., Verstraete, W., 2000. Probiotic bacteria as biological control agents in aquaculture. Microbiol. Mol. Biol. Rev. 64 (4), 655–671.

ISBN: 978-82-575-1660-4
ISSN: 1894-6402

Norwegian University
of Life Sciences

Postboks 5003
NO-1432 Ås, Norway
+47 67 23 00 00
www.nmbu.no

110360 / ANDVORDGRAFISK.NO