



Preventive Measures in Aquaculture
Isolation, Application and Effects of Probiotics on Atlantic
Cod (*Gadus morhua* L.) Rearing at Early Stages

Hélène L. Lauzon

Thesis for the degree of Philosophiae Doctor
University of Iceland
Faculty of Medicine
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December 2010

To my family, Sólveig and Guðmundur:

People who changed my world

Forvarnir í þorskeldi

Einangrun, notkun og áhrif bætibaktería á fyrstu stigum þorskeldis

Hélène L. Lauzon

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ÁGRIP

Léleg afkoma á fyrstu stigum stríðeldis er vandamál við framleiðslu þorskseiða (*Gadus morhua* L.) en á undanförunum árum hefur notkun sýklalyfja verið helsta úrræðið. Notkun bætibaktería er talin vera mögulegur valkostur sem fyrirbyggjandi aðferð til að hindra vöxt tækifærissýkla og sjúkdómsvaldandi baktería, og stuðla að stöðugleika í eldisumhverfi og bættri heilsu eldisdýra. Megintilgangur þessa rannsóknaverkefnis var að auka lifun og stuðla að þroskun þorsklirfa á fyrstu eldisstigum.

Í fyrsta hluta verkefnisins var hannað skimunarferli til að velja bætibakteríur úr eldisumhverfi þorsks. Niðurstöður sýna að 14% rannsakaðra baktería (n=188) höfðu hindrandi eiginleika gagnvart þremur bakteríum sem sýkja fisk, *Vibrio anguillarum*, *Aeromonas salmonicida* ssp. *achromogenes* og *Aliivibrio salmonicida*. Flestar valdar bakteríur voru Gram-jákvæðar (81%) og tilheyrðu Firmicutes (69.2%) og Actinobacteria (11.5%) fylkingum samkvæmt 16S rRNA hlutaraðgreiningu. Einungis sex (3.2%) rannsakaðra baktería hindruðu alla þrjá fisksýklana. Mismunur var á styrk og eðli hindrunar hjá bakteríunum. Fimm þeirra voru valdar til áframhaldandi rannsókna. Mikilvægt er að þróa blöndur af bætibakteríum til þess að auka líkur á áhrifaríkum forvörnum.

Í fyrsta tilraun var blanda þriggja valinna baktería notuð við reglulega böðun þorskhrögna og lirfa. Tvær þeirra, *Arthrobacter bergerei* (einangruð úr þörungabykkni) og *Enterococcus thailandicus* (einangruð úr eldisvökva þorsklirfa), fundust við lok tilraunarninnar í meðhöndluðum lirfum sem höfðu aukna lifun, meiri vöxt og lífsprótt auk takmakaðrar örveruflóru. Aðrar bætiörverur voru notaðar við auðgun hjóldýra sem notuð voru við startfóðrun lirfa, en sú meðhöndlun olli mikinn dauða hjá lirfunum og þeim tilraunum var því hætt. Einnig voru áhrif mismunandi meðhöndlunar á ræktanlega og ríkjandi örveruflóru þorskeldisins á tveimur klaktímabilum skoðuð. Þetta voru bæði aðferðir sem eru í notkun (sótthreinsum hrogn og lyfjameðferð) og nýjar aðferðir byggðar á notkun bætibaktería við meðhöndlun hrogn og lirfa eða hjóldýra. Ennfremur voru nokkrir eiginleikar tengdir sýkingarmætti rannsakaðir hjá bakteríum sem voru einangraðar úr eldisumhverfinu. Niðurstöðurnar sýna að bæði fóðrun og mismunandi meðferðir höfðu áhrif á örveruflóru í meltingarvegi lirfa og einnig að samsetning örveruflórunnar tengist velgengni í lirfueldi.

Í seinni hluta verkefnisins voru nefndar tvær bakteríur aftur notaðar við böðun hrogn og lirfa. Þetta var gert til að staðfesta getu þeirra til að efla forvarnir á fyrstu stigum í þorskeldi. Til viðmiðunar voru notaðar ómeðhöndlaðar eldiseiningar og aðrar sem fengu

hjöldýr meðhöndluð með bætibakteríublöndu sem er á markaði (Remus®). Niðurstöðurnar gefa til kynna að *A. bergerei* og *Ent. thailandicus* séu bætibakteríur, því þær hafa áhrif á örveruflóru í meltingarvegi lirfa og stuðla að vexti, þroskun og bættri meltingu lirfa með beinum eða óbeinum hætti.

Að lokum var framkvæmd rannsókn í seiðaeldi til að staðfesta eiginleika þessara tveggja baktería sem bætibaktería. Seiðum var gefið meðhöndlað þurrfóður í 28 eða 55 daga tímabil. Þurrfóðrið var úðað með bætibakteríunum (10^{7-9} CFU g⁻¹) og lífvænleiki þeirra kannaður við 8 vikna geymslu við mismunandi hitastig (-20, 4 og 15 °C). Áhrif bætibakteríanna á gæði fóðurs voru líka metin. Síðan voru metin áhrif mismunandi fóðurs (ómeðhöndlaðs eða meðhöndlaðs með *Ent. thailandicus* og/eða *A. bergerei*) á vöxt og lifun seiða. Ennfremur var fóðurstuðull metinn og örveruflóra eldisvökva og seiða könnuð. Í ljós kom að seiðin sem fengu Enterococcus-fóðrið uxu marktækt hraðar og höfðu marktækt lægri fjölda *Vibrio* tegunda í meltingarveginum en ómeðhöndluðu seiðin ($P < 0.05$).

Heildarniðurstöður rannsókna benda til þess að *Ent. thailandicus* og *A. bergerei* séu bætibakteríur, sem hafi áhrif á örveruflóru meltingarvegs þorsks á fyrstu stigum eldis og stuðli að auknum vexti og lifun.

Lykilord: Þorskeldi, bætibakteríur, örveruflóra, tækifærissýklar, lifun, þroskun lirfa, fóðrun seiða

ABSTRACT

Survival problems are encountered at early stages of intensive fish rearing and antibiotics are widely used to remedy the situation. Probiotics may provide a potential alternative method to protect larvae from opportunistic and pathogenic bacteria and promote a balanced environment. This research work was undertaken to promote survival and larval development at early stages of Atlantic cod (*Gadus morhua* L.) rearing.

The first part of the study was designed to search for probiotics to target this critical period in cod rearing. Potential probionts were selected from the natural microbiota of cod larviculture at an experimental station in Iceland, based on several *in vitro* tests. Our study demonstrated that 14% of screened bacteria (n=188) had antagonistic properties towards fish pathogens. The majority of the isolates were Gram-positive (81%), belonging to Firmicutes (69.2%) and Actinobacteria (11.5%) phyla based on 16S rRNA gene sequencing. Only six (3.2%) of 188 isolates could inhibit all three pathogens tested, *Vibrio anguillarum*, *Aeromonas salmonicida* ssp. *achromogenes* and *Aliivibrio salmonicida*. Differences observed in activity intensity and spectrum among inhibitory isolates emphasise the need to develop probiotic mixtures for efficient prophylactic methods.

Successively, three bacterial strains were tested *in vivo* as a mixture added to the rearing water from an early postfertilisation stage in the experimental cod hatchery. Two of the added bacteria (*Arthrobacter bergerei* isolated from an algal concentrate and *Enterococcus thailandicus* from cod larval rearing water) were re-isolated in larvae at the end of the experiment, contributing to increased survival, growth, vitality and microbiota control. A different microbial rotifer treatment was attempted to introduce putative probiotics during larval first-feeding but was not successful and led to high larval mortality. Further, the dominant culturable microbiota was characterised as influenced by specific treatments applied during two spawning seasons. Current hatchery practices (ova disinfection and antibiotic treatment of unhealthy larvae) were evaluated, as well as specific putative probionts either applied to ova and larvae or to rotifers as the delivery vector to larvae. Further, virulence-related phenotypic traits, including haemolysin, siderophores and AHLs, were studied among bacterial isolates and related to larval survival. The results clearly demonstrate the influence of exogenous feeding and treatments on larval gastrointestinal microbiota, and the role of bacteria in larval survival.

During the third spawning season, the combined application of the two prospective probionts was validated by their addition via the rearing water from the prehatch stage till 28-dph larvae. This bacterial bathing treatment was compared to groups fed rotifers supplemented with a commercial probiotic (Remus[®]) and those untreated. Effects of treatments on the microbial load in rearing systems, larval growth, survival and development were evaluated. The results suggest that *Arthrobacter* and *Enterococcus* probionts affected the larval GI microbiota and contributed to growth, development and digestion, either directly or indirectly.

Finally, an intervention study was conducted at an early cod juvenile stage to confirm the properties of the two probionts applied as a mixture or singly via dry feed (10^{7-9} CFU g⁻¹) to older fish for a shorter (28 days) or longer (55 days) period. This involved the development of probiotic feed, its viability and quality assessment under different storage conditions (-20, 4 and 15 °C), as well as the evaluation of effects on juvenile growth, survival, feed conversion ratio, microbial load of rearing water, juvenile gills and gut. Juveniles fed the *Enterococcus*-probiotic feed grew significantly faster and had significantly lower gut *Vibrio* counts than control juveniles ($P<0.05$). Overall, the results suggest that both probionts, especially the *Enterococcus* strain, modified the gut microbiota and contributed to enhanced juvenile growth and survival.

Keywords: Cod aquaculture, probiotic, microbiota, opportunistic bacteria, survival, larval development, juvenile feeding

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DECLARATION OF CONTRIBUTION

I was responsible for the conception and management of the Icelandic project “Preventive measures in cod aquaculture”, on which the thesis is based. The project (2004-2007) was a joined effort of three Icelandic institutes: the Icelandic Fisheries Laboratories (IFL)/Matís ohf, the Institute for Experimental Pathology (Keldur) of the University of Iceland and the Aquaculture Research Station of the Marine Research Institute (MRI). I therefore had a major role in the study design and execution of the experiments, in the acquisition of most data, their analysis and interpretation as well as in the structuring and writing of all the manuscripts.

I particularly worked at the cod microbiota isolation, enumeration and characterisation, and at the determination and selection of the putative probionts from the cod aquaculture environment. I designed and prepared the bacterial and microbial treatments tested in the various studies, performed the microbiological sampling and analysis throughout all experiments, as well as the larval viability counting and dry weight determination. I further evaluated the enzymatic profile of the prospective probionts and acquired most of the data relating to the juvenile trials.

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LIST OF ABBREVIATIONS

AHL	Acylated homoserine lactone
AP	Alkaline phosphatase
ApoLP	Apolipoprotein
ARA	Arachidonic acid (C20:4n-6)
ASW	Artificial seawater
ASWP	Artificial seawater supplemented with 0.1% (w/v) peptone
BA-NaCl	Blood agar containing 2% (w/v) sodium chloride
BBM	Brush border membrane
BF	Bluegill Fin
C3	Complement factor 3
C6-HSL	N-hexanoyl-L-homoserine lactone
3-oxo-C6-HSL	3-oxo-N-hexanoyl-L-homoserine lactone
CAS	Chrome azurol S
CdV41	<i>Carnobacterium divergens</i> V41
CFC	Cetrimide-Fucidin-Cephalordine (Pseudomonad medium)
CFU	Colony-forming units
CHSE	Chinook Salmon Embryo
COD	Chemical oxygen demand
CRP	C-reactive protein
CV026	<i>Chromobacterium violaceum</i> CV026
DGGE	Denaturing gradient gel electrophoresis
DHA	Docosahexaenoic acid (C22:6n-3)
DMC	Direct microscopic count
DNA	Deoxyribonucleic acid
DPA	Docosapentaenoic acid (C22:5n-6)
dpf	Day postfertilisation
dph	Day posthatch
DRBC	Dichloran rose Bengal chloramphenicol agar
EPA	Eicosapentaenoic acid (C20:5n-3)
EPC	Epithelioma papulosum Cyprini
FAA	Free amino acids
FBS	Foetal bovine serum
FCR	Feed conversion ratio
FISH	Fluorescence <i>in situ</i> hybridisation
GI	Gastrointestinal
GIT	Gastrointestinal tract
HUFA	Highly unsaturated fatty acids
Ig	Immunoglobulin
IgD	Immunoglobulin D
IgM	Immunoglobulin M
LAB	Lactic acid bacteria
LAMP	Loop-mediated isothermal amplification
LGG	<i>Lactobacillus rhamnosus</i> GG
lx	Lux
MA	Marine agar 2216
MB	Marine broth 2216
MLSA	Multilocus sequence analysis

MRI	Marine Research Institute
mRNA	Messenger ribonucleic acid
NAP	Nitrite actidione polymyxin agar
NL	Neutral lipids
OD	Optical density
OTU	Operational taxonomic unit
PA55/LDPE60	Polyamide 55 microns/low density polyethylene 60 microns
PAA	Protein-bound amino acid
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
ph	Posthatch
PL	Phospholipids
ppm	Part per million
PUFA	Polyunsaturated fatty acids
pZLR4	<i>Agrobacterium tumefaciens</i> NT1
QQ	Quorum quenching
QS	Quorum sensing
rpm	Revolution per minute
<i>rpoB</i>	RNA polymerase beta subunit
RTG	Rainbow Trout Gonad
RT PCR	Real-time polymerase chain reaction
RW	Rearing water
16S rDNA	16 subunit ribosomal deoxyribonucleic acid
16S rRNA	16 subunit ribosomal ribonucleic acid
S _A or S ₁	Silo A, silo 1, etc.
SAP	Serum amyloid P
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SGR	Specific growth rate
sp. or spp.	Species (for singular or plural term)
ssp.	Subspecies
T ₁ or T ₂	Tank 1 or tank 2
TCBS	Thiosulphate-citrate-bile-sucrose agar
TMS	Tricaine methane sulfate
TN	Kjeldahl nitrogen
TP	Total phosphorus
t-RFLP	Terminal restriction fragment length polymorphism
TTGE	Temporal temperature gradient gel electrophoresis
TVC	Total viable count
VBNC	Viable but non-culturable
v/v	Volume per volume
v/w	Volume per weight
ω-6	Omega-6
WB	Western blot
wph	Week posthatch
w/v	Weight per volume

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LIST OF ORIGINAL PAPERS

This thesis is based on the following original papers, which are referred to in the text by their Roman numerals (I-V):

- I. Lauzon, H.L., Gudmundsdottir, S., Pedersen, M.H., Budde, B.B., Gudmundsdottir, B.K. Isolation of putative probionts from cod rearing environment. *Veterinary Microbiology* 2008; 132 (3-4): 328-339.
- II. Lauzon, H.L., Gudmundsdottir, S., Steinarsson, A., Oddgeirsson, M., Petursdottir, S.K., Reynisson, E., Bjornsdottir, R., Gudmundsdottir, B.K. Effects of bacterial treatment at early stages of Atlantic cod (*Gadus morhua* L.) on larval survival and development. *Journal of Applied Microbiology* 2010; 108 (2): 624-632.
- III. Lauzon, H.L., Gudmundsdottir, S., Petursdottir, S.K., Reynisson, E., Steinarsson, A., Oddgeirsson, M., Bjornsdottir, R., Gudmundsdottir, B.K. Microbiota of Atlantic cod (*Gadus morhua* L.) rearing systems at pre- and posthatch stages and the effect of different treatments. *Journal of Applied Microbiology* 2010; 109 (5): 1775-1789.
- IV. Lauzon, H.L., Magnadottir, B., Gudmundsdottir, B.K., Steinarsson, A., Arnason, I.O., Gudmundsdottir, S. Application of prospective probionts at early stages of Atlantic cod (*Gadus morhua* L.) rearing. *Aquaculture Research* 2010; 41 (10): e576-e586.
- V. Lauzon, H.L., Gudmundsdottir, S., Steinarsson, A., Oddgeirsson, M., Martinsdottir, E., Gudmundsdottir, B.K. Impact of probiotic intervention on microbial load and performance of Atlantic cod (*Gadus morhua* L.) juveniles. *Aquaculture* 2010; 310 (1-2): 139-144.

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1 INTRODUCTION

1.1 Overview on current status of cod aquaculture

Progress in aquaculture over the last decades has led to the increased contribution of farmed fish to the fish consumption by the human population worldwide, accounting for 47% of the world's fish food supply in 2006. In fact, world aquaculture production in 2006 dominated in freshwater fishes (54%) compared to a low marine production (3%) (FAO, 2009). Owing to the fact that growth of human population is predicted to increase in the coming decades, and the aquaculture industry is growing faster than livestock production while wild catch volume has been stagnant or declining in recent years, a competitive and increased production of farmed fish is inevitable if current per capita consumption levels have to be maintained. However, a closer look at the aquaculture growth in terms of regions of the world or fish species reveals its ununiformity (FAO, 2009). Difficulties encountered in aquaculture developments may have arisen due to various constraints, either microeconomic, knowledge, technological, environmental or social. Problems like larvae or juvenile shortage, unstable production volumes, and uncertain profitability due to high production costs and unstable prices are currently faced by fish farmers dealing with fish species under development (Paisly *et al.*, 2010).

1.1.1 Production and development of cod aquaculture

Diversification of fish species in aquaculture production can contribute to the creation of a sustainable aquaculture sector. Atlantic cod (*Gadus morhua*) is a coldwater marine species with declining stocks, and being an important economic commodity in international markets, its candidature for aquaculture has motivated few countries around the North Atlantic Ocean. In the recent decades, much effort has been spent on developing commercial cod farming in Norway, Canada and the United Kingdom (UK) as well as in Iceland. Table 1 presents the cod production trends for farmed juveniles in these countries between 2003 and 2008, some of them being also involved in captured-based cod farming. Norway is the most important cod producer, with cod farming ranking in third place in Norway in terms of quantities but accounting for only 1.6% of their total aquaculture production (Paisly *et al.*, 2010). Based on these values, cod farming is still in its infancy, with a production in 2006 representing about 0.18% of the world marine aquaculture production (7.1 million tonnes, excluding China)

(Rana, 2007). Expansion of cod culture is apparently hampered by biological and technological constraints (FAO, 2009; Rana, 2007).

Table 1. Quantity^a in metric tonnes of slaughtered farmed cod from producing countries

Countries	2003	2004	2005	2006	2007	2008	Percentage of total production in 2008
Norway ^b	946	1685	5519	10384	10375	16523	87.3%
(incl. wild)	2185	3165	7409	11087	11104	18052	84.2%
Iceland ^c	0	0	169	180	525	517	2.7%
(incl. wild)	393	595	1050	1412	1467	1502	7.0%
UK ^d	82.1 ^f	8 ^f	69.6	543	1111	1822	9.6%
Canada ^e	0	0	0	1	15	75	0.4%
Total	1028	1,693	5757.6	11108	12026	18937	
(incl. wild)	2660.1	3768	8528.6	13043	13697	21451	

^a Numbers refer to quantity of cod (mt) produced commercially from juveniles, with a total production volume including capture-based cod farming (wild) as practiced in some countries; ^b Norwegian Directorate of Fisheries (NDF, 2010); ^c Jónsson (2010) and written communication (2010); ^d Anonymous (2009); ^e Frank Powell, Cooke Aquaculture (written communication, 2010); ^f Anonymous (2006).

Rearing of Atlantic cod has been conducted for over 100 years in Norway, aiming solely at yolk-sac fry production to restock local wild populations (Rosenlund & Halldorsson, 2007). It was not until 1970-1980 that interest in farming cod to market size developed in Norway. Cod were reared from ova to mature fish for the first time in 1977 (<http://www.thefishsite.com/articles/163/the-atlantic-cod-the-potential-for-farming-in-shetland>; retrieved 13.06.2010). However, in the last decades, several attempts to engage in commercial cod farming have failed in Norway as indicated by the decreasing number of companies involved in cod production (Figure 1). Eventually, Norwegian efforts have led to a feasible control of the broodstock quality and some cod farms have been profitable (http://www.fisheries.no/aquaculture/aquaculture_species/aquaculture_species_cod/; retrieved 13.06.2010), as demonstrated by production volume in Table 1. It is noteworthy that Norwegian statistics show that 42% of fish losses in 2008 were due to mortality in the pens, and the total number of lost individuals (n=6053) most likely corresponded to 0.1% of those harvested, assuming a 2-3 kg fish in size (NDF, 2010).

UK first harvested farmed cod in 2000 and increased their output considerably from 2007. In 2008, seven companies were running 14 cod farming sites (Anonymous, 2009).

However, several challenges, such as availability of juveniles by improved survival rates, must be overcome by the Scottish cod industry to reach planned expansion of more than 5000 tonnes within the next five years (retrieved 13.06.2010; <http://www.marlab.ac.uk/Delivery/standalone.aspx?contentid=764>).

Canadian cod farming started in the late 1980's with wild caught cod being on-grown through the summer period in Newfoundland, and in late 1990's an experimental hatchery production was established in the province. Cod farming has been in a developmental stage in Newfoundland, with 51 licences in 2006 and 17 in 2008 (retrieved 13.06.2010; <http://www.fishaq.gov.nl.ca/stats>). From 2004, commercial farming by Cooke Aquaculture was initiated in Eastern Canada (retrieved 13.06.2010; http://www.fiskeriforum.no/uploads/media/COD_Canada.pdf). Production of farmed cod has been minimal in Canada.

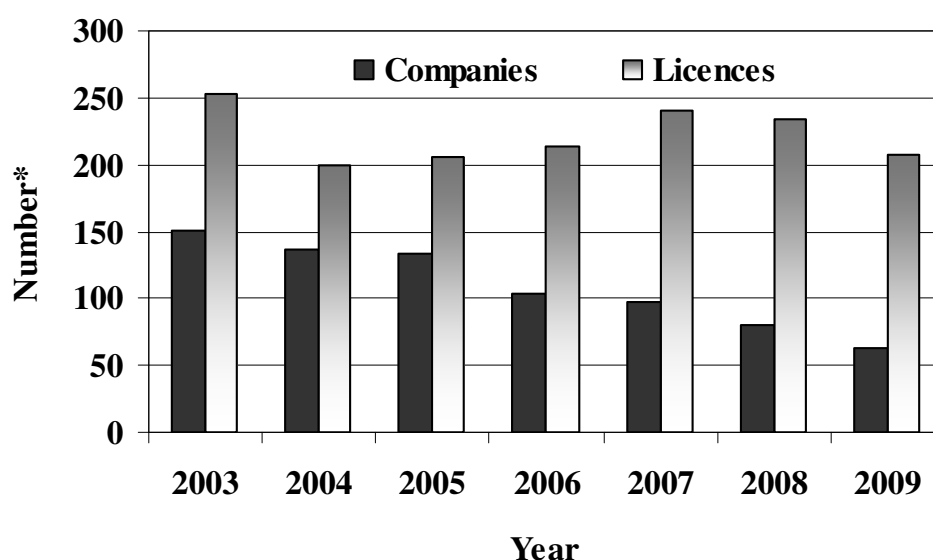


Figure 1. Number of companies and licences for cod production in Norway

* From Norwegian Directorate of Fisheries (NDF, 2010).

In Iceland, rearing of marine species started with Atlantic halibut (*Hippoglossus hippoglossus*) in 1987, followed by turbot (*Scophthalmus maximus*) and cod some years later (Paisly *et al.*, 2010). Capture-based cod aquaculture was first experimented in 1992 and continued on a limited scale until it expanded in 2002 upon governmental quota's allocation to promote cod aquaculture in Iceland (retrieved 13.06.2010; <http://www.fisheries.is/aquaculture/species/cod>). Meanwhile, experimental cod farming at the

Marine Research Institute (MRI) had been producing yearly about 5000 juveniles for research purposes since 1994 but in 2001, the output was doubled due to the reform on cod aquaculture development in Iceland. After a building extension at the MRI in 2003, juvenile annual production has been in the range of 200 to 250 thousands juveniles, with most of them being released to sea cages for commercialisation in Iceland and the Shetlands (Steinarsson, 2004). The company Icecod Ltd. initiated a selective breeding program in 2003 and started commercial juvenile production in 2008, while the MRI gradually withdrew from commercial production. Still captured-based cod aquaculture appears to be more economically efficient than using farmed juveniles (<http://www.fisheries.is/aquaculture/species/cod>; retrieved 13.06.2010). In 2009, cod farming was conducted in sea cages at eight sites around the country (Jónsson, 2010). Progress in the breeding program and hatchery production to deliver faster growing and healthy juveniles may lead to a considerable increase in volume in the years to come.

1.1.2 Problems to be solved: research needs and new trends

The challenges in farming new species are generally linked to research capacity, infrastructure, capital, production and environmental issues, as well as market issues. Proliferation of opportunistic and pathogenic microorganisms in intensive rearing systems is known to cause poor larval growth and high mortality rates (Munro *et al.*, 1995). For cod, high production costs and the lack of juveniles have led to the use of captured wild fish for farming purposes (Paisly *et al.*, 2010). However, difficulties have been encountered in capture-based farming in Iceland due to infection of newly captured juveniles and varying outcomes (Jónsson, 2010). In hatchery farming, several challenges must be met before a sustainable cod production can be achieved. Juvenile availability and quality are recognised as the main bottleneck and depend on intrinsic and extrinsic parameters. Variation in ova quality, especially from off-season populations, has influenced production outcome in Norway (Rosenlund & Halldorsson, 2007). A better understanding of the effect of broodstock nutrition and external cues on the reproductive performance and gamete quality must be sought (Bobe & Labbe, 2010). Extrinsic larval parameters may be controlled by fulfilling nutritional requirements, desirable environmental conditions and good farming practices. But this is not easily achieved since huge variations in survival and a high incidence of deformities have been reported in cod juveniles (Rosenlund & Halldorsson, 2007). These problems have in turn not been fully explained. Cultivated plankton, such as rotifers

(*Brachionus* sp.) and *Artemia*, are commonly used at first-feeding. Early larval weaning onto inert diets has been attempted to reduce the need for these live feeds since they are expensive and can be problematic due to their high bacterial loads and possible transmission of pathogenic or opportunistic bacteria. However, this has delayed larval growth (Baskerville-Bridges & Kling, 2000b), emphasising that a proper feeding strategy must be used (Callan *et al.*, 2003). This implies further research on larval nutritional requirements and means to reduce bacterial loads and to improve nutritional value of live feeds. Trials with natural zooplankton have demonstrated their superiority in fulfilling nutritional requirements of cod larvae in comparison to rotifers, resulting in significantly faster growth and less skeletal deformities (Imsland *et al.*, 2006). However, very few commercial hatcheries can rely on its availability. Similarly, formulated feed development with respect to particle size, nutrient balance and leakage as well as digestibility and palatability is of major interest because it will directly affect larval health and performance.

Health and disease management at early cod stages has much relied on the use of antibiotics since vaccination is only first meaningful for 1-g juveniles or bigger. The immune system of cod is not fully competent until 2-3 months posthatch (Magnadottir *et al.*, 2004; Schroder *et al.*, 1998). Before this stage, larvae must count on non-specific parameters for their defence against infection. Such parameters are phagocytes and humoral factors such as complement, lectins, lytic enzymes, antibacterial peptides and proteinase inhibitors (Magnadottir *et al.*, 2005). Modulation of the immune system following activation of these non-specific parameters may be achieved by a range of substances, generally of bacterial, fungal or plant origin (Magnadottir *et al.*, 2006). Research on immunostimulation of cod larvae and juveniles has been initiated but further work should be conducted (Magnadottir *et al.*, 2006; Pedersen *et al.*, 2004; Skjermo *et al.*, 2006). Health management in cod larviculture should be given priority. Use of antibiotics should be minimised to avoid the spread of antibiotic resistance genes in pathogenic bacteria as well as environmental contamination (Serrano, 2005). Instead, development of novel prophylactic methods should be encouraged.

1.2 Cod rearing and development at early stages of life

Cod hatchery production has been adapted from farming techniques developed in the Mediterranean for sea bass (*Dicentrarchus labrax*) and sea bream (*Sparus aurata*) that originated from Japan. This is mainly due to similarities encountered among these marine species with respect to the tiny size of ova and resulting larvae, rather undeveloped (Figure 2),

which first-feeding involves enriched live feed until fully weaned on a dry microdiet about two months posthatch.

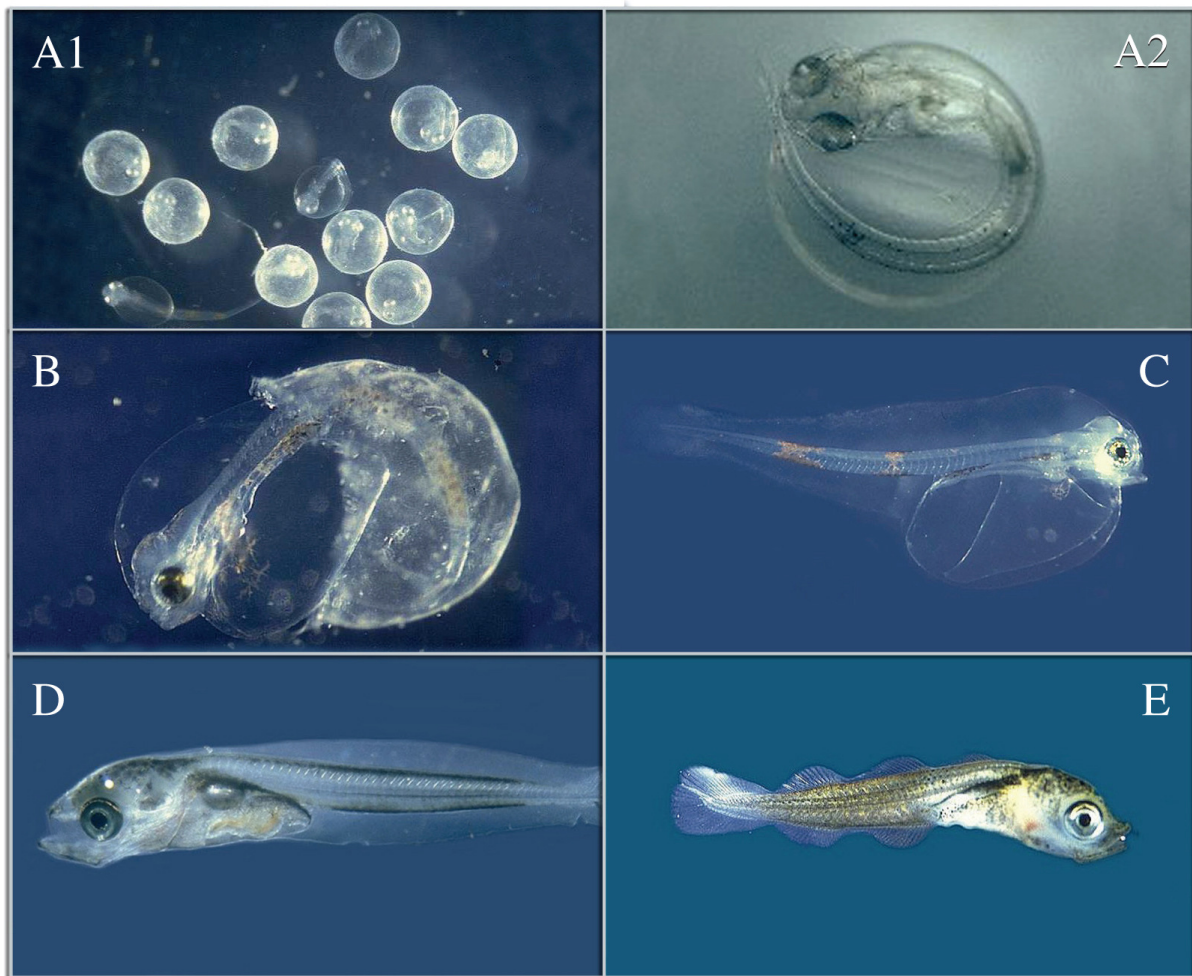


Figure 2. Cod larva from prehatch through metamorphosis

A₁₋₂, ova close to hatching; B, larva just hatching; C, newly hatched yolk-sac larva;

D, first-feeding larva with rotifers in gut; E, larva close to metamorphosis.

A₁-B-E, www.larvalbase.org/PhotoArchive, retrieved 5.12.2006

A₂-C-D, <http://notendur.hi.is/bergmagn/Gadus%20morhua/index.html>, retrieved 7.07.2010

Four successive developmental stages are recognised in cod culture: ova, larva (4-12 mm), early juvenile (12-45 mm) and juvenile (>45 mm). Metamorphosis occurs during the early juvenile period, with the disappearance of the continuous finfold and the formation of discrete fins, followed by the development of the spinal cord, stomach, pyloric caeca and mouth into the juvenile form (Pedersen & Falk-Petersen, 1992). A period of 16 weeks is generally required to obtain a 5-g juvenile, from the incubation of fertilised ova (2 weeks), followed by hatchery (8 weeks) and nursery (6 weeks) rearing (Steinarsson, 2004). Cod culture can be

performed extensively, semi-extensively and intensively. Intensive culture implies indoor hatcheries using rotifers and *Artemia* as live feed and formulated feed. However, it has been found to lead to decreased larval growth and the development of deformities compared to natural systems which are based on copepods grown in seawater lagoons (Imslund *et al.*, 2006). Nutrition, environmental conditions and early husbandry practices may all influence the poorer outcome in intensive rearing (Hamre, 2006). This section discusses the main cod stages as well as important parameters influencing intensive rearing.

1.2.1 Prehatch period

Availability and quality of ova are one of the prerequisites for successful farming. Cod ova can be procured in two ways, from wild spawning fish at sea on board a fishing vessel or from broodstock fish raised at the farm. Both ways have pros and cons. With wild ova, there is no need to raise broodstock fish which can be costly, quality and abundance are generally easily obtained, but the time period is restricted to the natural 2-month spawning season and genetic selection is not possible. It has also been shown that the earlier weeks of spawning harvest larger and higher quality ova (Marteinsdottir & Steinarsson, 1998).

In regard to broodstock, generally better fertility, selection of favourable genetic traits and availability all year round by photoperiod manipulation are attracting attributes (Steinarsson, 2004), but maintenance of several broodstock fish requires care and knowledge to harvest high quality ova (Rosenlund & Halldorsson, 2007). Penney *et al.* (2006a) found that photoperiod did not negatively affect fertilisation success or rates of cell developmental abnormalities, but it had a negative effect on both volume and size of cod eggs produced. An inverse linear relationship was observed between egg diameter and temperature in cod manipulated by photoperiod (Buckley *et al.*, 2000).

Ova fertilisation can be achieved by incubating freshly stripped milt and ova for 10 min followed by ova rinsing with seawater (Steinarsson, 2004). Disinfection of ova is then carried out prior to silo incubation to reduce microbial contamination, especially pathogens either from the wild or broodfish environment. Colonisation and bacterial growth on fish ova can pose great problems as it can lead to low oxygen accessibility and death (Hansen & Olafsen, 1989). It is likely that the microbial community of the ambient environment of developing fish ova will influence their microbiota composition, but also the glycoproteinaceous nature of the eggshell will selectively favour colonisation of specific

bacterial groups. This explains the species-species differences observed between bacterial groups colonising cod and halibut ova (Hansen & Olafsen, 1989).

Optimal incubation conditions of fertilised cod ova with respect to temperature and density have been studied. A parabolic relation was found when assessing the effect of incubation temperature (2-13 °C) of cod ova to the resulting dry weight of newly hatched larvae, with the optimal temperature defined as 7.6 °C (Steinarsson, 2004). Importantly, cod larval dry weight at hatch has been found to positively correlate to larval growth potential and survival (Marteinsdottir & Steinarsson, 1998). Moreover, the prevalence of malformed cod larvae with severe vertebral curvature was found to increase significantly as ova incubation temperature was raised from 6 to 11 °C (Fitzsimmons & Perutz, 2006). Puvanendran *et al.* (2009) demonstrated that the incubation density (3-48 mL ova L⁻¹) did not have any significant effects on vertebral deformities in Atlantic cod larvae, while increased incidences were observed at late larval stage indicating a possible nutritional or prey-type effect at metamorphosis. Further, they suggested that broodstock husbandry, genetics and/or nutrition could play a major role in causing vertebral deformities in cod larvae at hatch. Fertilised cod ova hatch approximately 12-14 days after fertilisation as influenced by incubation temperature and treatment.

1.2.2 Posthatch period: from a larva to an early juvenile

Cod larvae are small (3.3-4.5 mm in length) at hatch with a large yolk sac, no hemoglobin or gills, a simple intestinal tract, several non-functional organs, undifferentiated skeleton, incompletely developed nervous system and sense organs. They have rudiments of jaw bones and the anus is closed (Falk-Petersen, 2005). Larvae receive nourishment from their yolk sac during the first 3-6 days of life. The nutritional value of yolk reserves is influenced by the dietary status of the mother fish and can be enhanced through maternal transfer (Izquierdo *et al.*, 2001). In response to an increased energy demand after hatching, lipid mobilisation occurs in larvae, providing them with energy for swimming (Kamler, 2008). About two days posthatch (dph), their mouth opens with functional jaws seen three days later (Falk-Petersen, 2005). As the yolk sac is depleted, the onset of exogenous feeding takes place. Proper nutrient input and optimal environmental conditions will influence larval development and further differentiation.

Larval density in cod rearing tanks generally ranges between 10-50 larvae L⁻¹ (Brown *et al.*, 2003). Higher densities may result in lower production costs, but deterioration of water

quality under such conditions is an important risk to consider. Alver *et al.* (2007) reared at a maximal density of 80 cod larvae L⁻¹ without observing adverse effects with respect to larval growth and survival. Baskerville-Bridges and Kling (2000c) examined a larger larval density range (50-300 larvae L⁻¹) and observed little difference in survival among the 50-200 larvae groups while a lower average survival was seen in the most dense group concomitantly to elevated ammonia levels in the poorest replicates.

Live prey size is an important parameter during first-feeding of cod larvae. The results of Puvanendran *et al.* (2004) suggest that cod larvae are gape-limited predators, selecting small rotifers from 5 dph while larger rotifers and *Artemia* can be ingested from 8 and 26 dpf, respectively. Further, prey density influences larval growth and survival with the appropriate ration around 100 preys for each larva, given three times daily (Puvanendran & Brown, 1999; Puvanendran *et al.*, 2002). Fastest rates of gut clearance in cod larvae occur at first-feeding and metamorphosis (Jordaan & Brown, 2003). The prey type can influence larval growth, deformities and quality of resulting juveniles. Imsland *et al.* (2006) evaluated different startfeeding diets, using either natural zooplankton or cultured rotifers, and demonstrated that zooplankton fed at larval stage can greatly improve long-term growth and quality of cod juveniles.

Other extrinsic factors, such as light and temperature, are known to improve larval health and development. Light was shown to increase cod metabolic rate by 30 to 40% in small larvae (4-7 mm) but not in larger juveniles (40-60 mm) (Finn *et al.*, 2002). Puvanendran and Brown (2002) found better larval growth and survival at high light intensities (1200-2400 lx) and 24-h light during 6 weeks (wph). Monk *et al.* (2006) studied the effect of a reduced light intensity at a later larval stage and found that reduction from 28 dph was appropriate since it enhanced larval growth. This less distracting environment apparently promoted larval foraging efficiency, reducing the need for swimming hence sparing energy for growth. These findings may be related to those of Vollset *et al.* (2009) who showed that the vertical behaviour of larval cod in a water column, with respect to temperature and light, changes with ontogeny. Briefly, an early surface oriented behaviour changed to a deeper distribution with age.

At first-feeding stage, cod larvae are generally maintained at temperature close to ova incubation, but upon feeding, the temperature is increased by approximately 1 °C daily until 10 to 12 °C is reached. Such temperature regime is generally followed since larval growth is temperature- and size-dependent, and increasing temperature will reduce larval stage duration. However, moderate temperature should be maintained to avoid bacterial problems. Maximum

growth rate occurs in larvae in the dry weight range of 0.1-1.0 mg, after which a declining trend is observed especially at rearing temperatures of 8-14 °C (Otterlei *et al.*, 1999). Steinarsson and Bjornsson (1999) reported a maximum growth rate of 12-13% day⁻¹ at 13 °C in cod larvae fed live feed while Otterlei *et al.* (1999) observed a higher rate (18-20% day⁻¹ at 12-14 °C) in larvae (<12 mm) fed natural zooplankton. It is important to maximise larval growth at early stage since growth differences during first-feeding of cod have been shown to persist after 30 months in sea pens (Imslund *et al.*, 2006). Larval respiration rate is exponentially related to temperature, but with increasing body size the dependency of cod metabolism on temperature decreases. This may partly explain why juveniles can grow well over a broader range of water temperatures than larvae (Peck & Buckley, 2008).

At this early developmental stage characterised by rapid growth, fish larvae have a high dietary requirement for amino acids in response to the increase in body muscle mass by protein synthesis and accretion (Ronnestad *et al.*, 1999) and because free amino acids (FAA) are the major larval energy source (Finn *et al.*, 1995; Finn *et al.*, 2002; Fyhn *et al.*, 1987). Since cod stomach development is not completed until after metamorphosis (about 50 dph), early larvae have a digestive system with low proteolytic and absorptive capacities at first-feeding. Sveinsdóttir *et al.* (2006) reported minimal activity of digestive enzymes (trypsin and chymotrypsin) just prior to cod first-feeding, emphasising the insufficient digestive function in larvae at this stage. *In vivo* studies at early stages of marine fish larvae have shown higher absorption of FAA than peptides and protein-bound amino acids from the larval gut (Rust *et al.*, 1993). Lipid absorption occurs via the epithelial cells of the foregut and midgut while pinocytotic activity takes place in the hindgut. The first signs of intestinal absorption have been observed around 7-9 dph (Falk-Petersen, 2005).

Wold *et al.* (2007) observed in cod larvae that the specific activity of alkaline phosphatase (AP), a brush border membrane (BBM) differentiation marker, increased readily between 24 and 45 dph, demonstrating intestinal maturation. This coincides with the findings of Kvale *et al.* (2007) who suggested a matured intestine to be attained in cod larvae 43-53 dph. The earlier part of the 24-45 dph period corresponds to the peaking allometric growth seen by Finn *et al.* (2002) in 7-12 mm cod larvae. In fact, Wold *et al.* (2008) showed that the length and volume of the midgut epithelium were positively related to larval standard length, with a considerable increase in total midgut microvilli surface in 10-14 mm cod larvae. This explains why early weaning of cod larvae (<12 mm) preceding this period has led to poor larval growth and survival (Baskerville-Bridges & Kling, 2000a; Skjermo *et al.*, 2006; Stoss *et al.*, 2004; Wold *et al.*, 2008). Diet composition may stimulate the activity of digestive

enzymes and should be considered in feed development. Indeed, enzymatic activity has been found to significantly increase in cod larvae fed high lipid-enriched rotifers (O'Brien-MacDonald *et al.*, 2006).

1.2.3 Juvenile period

Weaning is an important step in the feeding process of early juveniles and generally completed at 8 wph (Brown *et al.*, 2003). Before early weaning of cod larvae can be successful, further improvements in formulation and development of artificial diets are essential since larvae appear to utilise such diets with lower efficiency than live prey. Artificial diets generally contain insoluble dietary components bound together with insoluble binders. Important characteristics of artificial diets include their water stability, palatability, nutritional value, digestibility as well as settling and leaching rate (Baskerville-Bridges & Kling, 2000a). Because of the slow development of the gastrointestinal tract (GIT), early cod juveniles may require dietary supplies of low-molecular weight peptides and amino acids that are more easily digested and absorbed than proteins. Dietary hydrolysed protein was shown to be advantageous in cod weaning diets (Kvale *et al.*, 2009), but leakage of low-molecular weight, water soluble nutrients can be problematic (Langdon, 2003). A shorter and more efficient weaning period may result in the reduction of live feed production costs. Puvanendran *et al.* (2006) evaluated the effect of temperature during cod weaning (from 45 dph) and suggested a possible optimal weaning temperature to be between 11.5 and 14.5 °C.

After weaning it is generally timely to size grade early juveniles because at this stage they grow rapidly and have a tendency to cannibalism and antagonistic behavior. This generally begins towards the end of metamorphosis and becomes a critical problem during the first 3-4 months as juveniles (Baskerville-Bridges & Kling, 2000c). Early juveniles are then grown for 6 weeks in nursery tanks from 0.3 to 5 g (wet weight). Immunisation by bathing against *Vibrio anguillarum* is performed twice during this period along with further size grading (Steinarsson, 2004). Because of handling stress caused to early juveniles, the use of immunostimulants is of particular interest. Stress may cause immunosuppression (Ellis, 2001) while prolonged used of immunostimulants may lower the immune status of the fish (Sakai, 1999). The effect of immunostimulants is mediated through non-specific immune mechanisms and the response period is limited. Pedersen *et al.* (2004) investigated whether oral administration of cationic cod milt proteins to cod juveniles (5 g) could improve disease resistance during a challenge trial with *V. anguillarum*. Their results suggest that feed

supplemented with the immunostimulant can improve juvenile resistance to this bacterial infection.

Optimisation of juvenile growth can be achieved by the selection of a proper temperature regime. Björnsson and Steinarsson (2002) evaluated the food-unlimited growth rate of cod juveniles (2 g to 5 kg) over a temperature range of 2-16 °C and found that temperature had a much greater effect on the growth rate of small juveniles than on that of larger ones. The non-linear growth rate model resulting predicts that the optimal temperature for cod growth decreases with increasing fish size, going from 12.8 °C for 50 g juveniles to 8 °C and 5.9 °C for 500 and 5000 g fish, respectively. Smaller juveniles, 2-20 g, appear to have an optimal temperature greater than 16 °C. This coincides with the findings of Imsland *et al.* (2006) who studied the effect of temperature (7-16 °C) on cod juveniles (growing from 9 to 40 g) and found that the temperature regime going from 16 to 10 °C over a 10-week period led to slightly better growth than the isothermal one at 16 °C.

1.2.4 Immune system of cod at early stages

Fish possess both an innate and adaptive immune system. The immune system in marine teleost fish is not completely developed during embryonic and larval stages and immune competence is assumed to be restricted. Innate parameters become crucial in immune defence of fish at early stages since their adaptive response becomes established later, being then essential for lasting immunity and a key factor in successful vaccination. All the basic features of the adaptive immune system are present in fish, but the antibody repertoire is more restricted in fish than mammalian species (Magnadottir, 2010). Further, fish adaptive response is slow and temperature-dependent in contrast to the innate immune system which is fast acting and relatively temperature independent (Magnadottir, 2006).

The main components of the innate immune system of fish are; (1) an epithelial/mucosal barrier, involving the skin, gills and GIT, (2) humoral parameters and (3) cellular components. Antimicrobial peptides, complement factors and immunoglobulins are part of the immune defence parameters of fish mucus. Humoral parameters include complement system, lectins, pentraxins (C-reactive protein, CRP; serum amyloid P, SAP), antibacterial peptides and protease inhibitors. Cellular components are phagocytic leukocytes and non-specific cytotoxic cells. Immunoglobulins (antibodies), expressed either as B-lymphocyte receptor or secreted in plasma, represent the humoral components of the adaptive immune system. Teleost fish produce only one functional immunoglobulin class, a tetrameric

IgM. The lymphocytes, the B- and T-cells, are responsible for specific pathogen recognition and initiation of the adaptive immune response (Magnadottir, 2010).

Magnadottir *et al.* (2004) monitored the ontogenic development of innate immune parameters of cod ova and larvae and detected the complement component C3 and the closely associated apolipoprotein (ApoLP) A-I from the time of embryo organogenesis, 7-9 days postfertilisation (dpf). Vitellogenin-derived maternal proteins, other non-vitellogenin maternal proteins and cathepsin were also detected in fertilized ova. Seppola *et al.* (2009) reported the presence of transcripts encoding lysozyme and cathelicidin in unfertilised cod ova, indicating maternal transfer of antibacterial transcripts, while hepcidin, pentraxin, ISG15 and LGP2 (two antiviral genes), were found to be transcribed during the early stages of embryogenesis. Lysozyme activity was also present at this stage, suggesting the presence of a functional protein and its role for survival during embryogenesis and larval development. Lysozyme is a bactericidal enzyme present in mucus, lymphoid tissue and serum of most fish species. The Ig heavy chain C-regions (IgM and IgD) were transcribed at a very low level during the ova segmentation period (from 5 dpf through hatching). Around hatch and first-feeding, most innate immune genes showed an increase in transcription, suggesting a preparation for increased pathogen exposure at this time.

Schroder *et al.* (1998) studied the appearance of cod lymphoid organs, detecting head kidney and spleen at hatch whereas thymus was observed few weeks later (9 mm larvae). Fully developed lymphoid organs were achieved after metamorphosis (49 dph in 25 mm larvae) and IgM first detected 58 dph in 33 mm larvae. This coincides with the findings of Seppola *et al.* (2009) where studied genes showed a pronounced elevation in transcript levels around and after cod metamorphosis, reflecting a further maturation of the immune system at this stage. Still, this late appearance of the primary humoral component of the adaptive immune system demonstrates the dependency of cod larvae on innate immune parameters for the defence against pathogens or opportunistic agents during their first 2-3 months of life. In fact, the first detection of IgM positive cells does not necessarily correlate with the ability to develop an antibody response to an antigenic stimuli (Magnadottir *et al.*, 2005). In juvenile cod, environmental temperature was shown to modulate their innate and adaptive humoral immune responses differently, the former being depressed and the latter increased by increasing temperature (Magnadottir *et al.*, 1999).

The complement system is important both in the innate and adaptive immune response, with C3 as the central protein of all three activation pathways. Using immunohistochemistry, Lange *et al.* (2004b) detected C3 in the yolk sac membrane from 1

dph, and in liver, brain, kidney and muscle from 2 dph, as well as in other organs such as eye, notochord, stomach, intestines, pancreas, heart and gills at different cod larval stages. Following the development of mRNA probes for cod C3 and ApoLP A-I, Lange *et al.* (2005) also detected C3 transcription in the central nervous system, skin and chondrocytes at different stages of development. ApoLP A-I was found in several of the above-mentioned organs. These findings suggest that complement is not only important in immune defence against invading pathogens but may also play a role in the formation and generation of different organs.

1.3 Microbiology of rearing systems

Until recently, most studies investigated the microbial load and microbiota composition of rearing systems using cultivation methods and microbiota identification relied on phenotypical and biochemical key characteristics (Alsina & Blanch, 1994a, b; Cahill, 1990). The complexity observed during bacterial identification in earlier days and the fact that bacterial classification has been revised intensively recently indicate that comparison with literature from the past will be difficult. In the last years, phenotypic traits or genotypic fingerprints have been used for tentative grouping to reduce the number of bacterial strains for sequence analysis of the 16S rRNA gene and phylogenetic identification. Some work has therefore focused on the isolation and characterisation of the dominant part of the culturable aerobic microbiota assuming that these microorganisms may play an important role *in vivo*. Less attention has been towards the anaerobic microbiota of fish intestine, but its low proportion has been reported in fish reared at low temperature (Léssel, 1990) and in increasing salt concentration (Cahill, 1990). It can therefore be expected to be of little importance in marine fish from coldwaters. Recently, researchers have used culture-independent methods to assess the abundance of the total microbiota and the composition of dominating bacteria. This tendency to apply non-cultivation methods rather than cultivation is because marine bacteria may enter a viable but non-culturable (VBNC) state, in which they no longer grow on conventional culture media, but remain intact and retain metabolic activity (Roszak & Colwell, 1987). Moreover, it has been shown that VBNC cells remain potentially pathogenic (Colwell *et al.*, 1985).

1.3.1 Microbiological detection and identification methods

Methods to detect, enumerate and identify microorganisms are widely described and may be based on a conventional or molecular approach. However, either approach has both advantages and disadvantages, which may be the basis for the selection of a method as well as its feasibility and cost, in addition to the knowledge and equipment required.

Conventional plate count methods are commonly used to isolate and enumerate viable microorganisms and generally involve sample preparation, dilution, homogenization, serial dilution, plating of aliquots, media incubation and enumeration of visible colonies, reported as colony-forming units (CFU). Several factors may influence the outcome, namely the appropriateness of sample storage and preparation conditions, microbiological media and incubation conditions used, as well as the presence of competing or antagonistic microorganisms (Jay, 1992). Any condition undesirable to certain microorganisms may inhibit their growth and detection despite their presence and culturability. For instance, chilled diluent, spread plating and longer incubation time at lower temperature are recommended for the evaluation of fish psychrotrophic bacteria (Dalgaard *et al.*, 1997). Heat labile and sodium requiring microorganisms are common in seawater, emphasising the importance of a proper methodology. Seafood analysis has shown that spread plating may result in about twenty times higher total viable counts compared with pour plating (Lopez-Caballero *et al.*, 2002). Further, Schulze *et al.* (2006) demonstrated that a wide bacterial diversity of the culturable microbiota in a marine hatchery can be obtained using several media types. Another limitation is related to the presence of VBNC microorganisms which due to their state (stressed or nutrient starved) may not be able to grow despite the proper incubation conditions. Also, some microorganisms have been classified as non-culturable since their cultivation has not been achieved yet (Amann *et al.*, 1995). Further, the use of selective media may allow for the selection of specific microorganisms, but depends readily on the degree of inhibition and effectiveness of the differential agents used. For this reason, it is necessary to verify the composition of the selected microbiota to confirm the medium selectivity.

The culturability of bacteria in water environments has been reported to be below 1% of total microbiota (Amann *et al.*, 1995). Total direct counts of bacterial abundance in such environments have been accomplished using direct microscopic counts (DMC) with fluorochrome staining of bacteria but such counts generally include both viable and non-viable cells. Overestimation of bacterial cell abundance has been reported in lower cell density samples ($<10^5$ filter⁻¹), indicating the limitation of this method for samples with debris (Lisle *et al.*, 2004). Spanggaard *et al.* (2000) investigated the microbial load of rainbow trout

(*Oncorhynchus mykiss* Walbaum) intestine and found that plate counts achieved in average 50% of total microbiota assessed by DMC. Nutrient-rich environments, such as soiled rearing water, live feed culture, fish ova and gut, have been shown to harbour high levels of culturable bacteria (Huber *et al.*, 2004; Lopez-Torres & Lizarraga-Partida, 2007; Salvesen *et al.*, 1999; Verner-Jeffreys *et al.*, 2006). Further, Pinhassi *et al.* (1997) reported a good agreement between cultivation method and DMC when assessing the number of nucleoid-containing cells in bacterioplankton samples. Using nucleic acid dyes for DMC, Huang *et al.* (2005) showed that it was possible to distinguish between viable, injured and non-viable bacterial cells.

Molecular methods based on the polymerase chain reaction (PCR), rDNA sequence analysis and fluorescence *in situ* hybridisation (FISH), have been used in microbial ecology for identification and specific enumeration of bacteria. Specific 16S-rRNA-targeted oligonucleotide probes can detect and enumerate phylogenetic groups among the bacterial community using FISH, but variable probe specificity has been reported (Huber *et al.*, 2004; Lopez-Torres & Lizarraga-Partida, 2007). Denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rRNA genes of total bacterial DNA from an environmental sample has been used to visualise the bacterial diversity as a DNA fingerprint. This banding pattern is achieved in an agarose gel, which can be cut for band sequencing and phylogenetic identification. Brunvold *et al.* (2007) found such a DGGE method to be suitable for characterisation of bacterial communities in cod hatcheries, but pointed out that other genes might be more suitable for the discrimination of closely related taxa, particularly different *Vibrio* spp. Bias in molecular methods may be related to the variable efficiency of cell lysis, DNA extraction and purification as well as PCR amplification. With the mentioned DGGE method, bands at the same position in a gel have the same melting behaviour, but not necessarily the same sequence (Muyzer *et al.*, 1993). Also, the problem of heterogeneity between copies of 16S rDNA on the bacterial genome may lead to multiple bands from one bacterium. However, the use of a non-heterogeneous gene, like *rpoB*, in PCR-DGGE and TTGE (temporal temperature gradient gel electrophoresis) based community analysis has been shown to remedy this problem (Dahllöf *et al.*, 2000; Navarrete *et al.*, 2010). Population fingerprinting can also be achieved by terminal restriction fragment length polymorphism (t-RFLP), but bacterial diversity may be underestimated because unrelated bacteria may share the same terminal restriction site and result in identical DNA fragments (Kent *et al.*, 2003). Another PCR-based strategy is 16S rRNA clone analysis which, based on the number of clones sequenced, can provide a more detailed microbiota composition as well as its relative

proportion (Schmidt *et al.*, 1991). Multilocus sequence analysis (MLSA) is a typing tool based on housekeeping genes, and using several of these markers will enhance the discriminatory power to separate 16S rRNA closely related species, like vibrios (Sawabe *et al.*, 2007; Thompson *et al.*, 2005).

Conventional cultivation methods, biochemically and serologically based systems, like API 20E, BIOLOG, monoclonal antibody based methods and sandwich techniques, as well as immunohistochemistry have been used to diagnose fish pathogens (Austin, 1998). However, the major drawback of these techniques is their limited sensitivity. The rapid detection and identification of pathogens is crucial for effective disease management in aquaculture. Real-time (RT) PCR has been used for specific detection of target bacteria as well as their relative quantification in aquaculture (Balcazar *et al.*, 2007; Fujita *et al.*, 2006; Panangala *et al.*, 2007; Soto *et al.*, 2010; Suzuki & Sakai, 2007). Loop-mediated isothermal amplification (LAMP) is an alternative method that, like PCR, is based on DNA amplification. This rapid and sensitive method was recently developed for the detection of *Aeromonas salmonicida* (Kulkarni *et al.*, 2009), *V. anguillarum* (Gao *et al.*, 2010) and *Francisella piscida* (Caipang *et al.*, 2010b), causing atypical furunculosis, vibriosis and francisellosis, respectively, in cod. This method requires a minimal equipment and analysis time, and could therefore potentially be applied to the screening of pathogens in aquaculture farms.

1.3.2 Microbiota of cod rearing systems: quantitative and qualitative evaluation

High mortality rate at early larval stage in intensive cod hatcheries is common. Several factors may play a role, such as suboptimal rearing conditions, nutritional status and immature immune system of larvae as well as detrimental or pathogenic microorganisms. Apart from the invasion of recognised pathogens, poor larval viability may be caused by opportunistic bacteria which become harmful under the circumstances created, due to the weakening of host resistance resulting from environmental stress and/or detrimental bacteria (Skjermo *et al.*, 1997). Bacterial interaction at mucosal surfaces of ova and larvae have been associated with the rearing success or failure (Hansen & Olafsen, 1999). Improved husbandry and health management practices may be achieved upon a better knowledge of the composition, origin and role of microbiota in rearing systems.

Hansen and Olafsen (1989) investigated the culturable cod ova microbiota which included *Pseudomonas*, *Alteromonas*, *Aeromonas*, *Flavobacterium*, *Cytophaga* and *Flexibacter* spp., as well as *Leucothrix mucor* and *Vibrio* (*Aliivibrio*) *fischeri* based on

phenotypic characterisation. Opportunistic vibrios were not preponderant on cod ova despite the presence of some *Vibrio* spp. in the rearing water. Bacterial colonisation of cod ova was detected as early as 2 h postfertilisation. Recently, 16S rDNA-based PCR-DGGE microbiota analysis of cod rearing reported the presence of *Colwellia* and *Pseudoalteromonas* spp. on ova (McIntosh *et al.*, 2008). Bergh (2000) screened 43 cod ova batches for the presence of *Tenacibacter ovolyticus*, a pathogen which was not found in contrast to its high occurrence on halibut ova. Treasurer *et al.* (2005) reported a culturable microbial load of 10^4 CFU mL⁻¹ for newly fertilised cod ova while no data is available on microbial accumulation during incubation. Microbial proliferation in rearing water of ova incubators was reported by Hansen and Olafsen (1989), increasing prior to hatch from 10^3 to 10^6 mL⁻¹ 2 dph.

Healthy ova hatch sterile larvae, but very soon after hatching and before the introduction of live feed, they become colonised and their intestinal microbiota corresponds to that found on ova surface (Hansen & Olafsen, 1989; Skjermo *et al.*, 2006). Brunvold *et al.* (2007) reported that GIT of 5-dph cod larvae fed algal culture was associated with gamma-Proteobacteria (*Alteromonas* sp.) followed by the introduction of alpha-Proteobacteria upon rotifer feeding. Indeed, a bacterial succession as larval stage progressed was observed in the study of Reid *et al.* (2009) and McIntosh *et al.* (2008), who generated a dynamic description of larval bacterial colonisation. The latter authors reported the dominance of *Ruegeria/Roseobacter*, *Pseudoalteromonas* and *Microbacterium* spp. in GIT of cod larvae fed on rotifers, but when the diet was changed to *Artemia* over 90% of the microbiota consisted of vibrios, *Vibrio alginolyticus* especially. Then weaning onto dry feed led to a population shift towards one dominated by *Vibrio splendidus*. The observed succession agrees with the findings related to other fish species (Blanch *et al.*, 1997; Eddy & Jones, 2002; Griffiths *et al.*, 2001; Jensen *et al.*, 2004; Verner-Jeffreys *et al.*, 2003). In more, Fjellheim *et al.* (2007) observed variations in bacterial composition among cod larvae, within and between groups studied. Culturable microbial load of cod larval gut amounted to 10^2 CFU larva⁻¹ early posthatch, while upon live feed introduction it reached 10^{5-6} CFU larva⁻¹ followed by a 10-fold reduction after weaning (Reid *et al.*, 2009; Strom & Ringo, 1993). The bacterial numbers in rearing water was shown to increase shortly after the introduction of both live feed and dry feed, and thereafter to decrease as a result of increased water exchange (Skjermo *et al.*, 2006).

Several studies have shown that larval gut microbiota is influenced by the diet. The study of Korsnes *et al.* (2006) on the effect of different rotifer enrichment media indicated their influence on the culturable GIT microbiota of cod larvae fed rotifers, both quantitatively and qualitatively. Interestingly, the enrichment diet containing a lower protein, carbohydrate

and lipid content, but a high docosahexaenoic acid (DHA, C22:6n-3) level and DHA/EPA (eicosapentaenoic acid, C20:5n-3) ratio, resulted in lower bacterial levels in the rotifer culture and larval gut. Nevertheless, *Vibrio* spp. were prevailing in the culturable microbiota associated with the differently enriched rotifer cultures. The culturable microbiota in rotifer mass cultures was reported to include *Vibrio*, *Pseudoalteromonas*, *Pseudomonas*, *Microbacterium*, *Micrococcus*, *Moraxella* and *Flavobacterium* spp. (Korsnes *et al.*, 2006; Skjermo & Vadstein, 1993; Verdonck *et al.*, 1994). Rotifers are filter-feeders taking up bacteria from their surrounding water, hence reflecting their microbiota. Rotifers, typically grown on baker's yeast and an oil source, may contain 10^{3-4} CFU per individual (Skjermo & Vadstein, 1993) in contrast to about 10^2 CFU per individual for those fed with microalgae (Oie *et al.*, 1994). Using a culture-independent method (DGGE), the microbiota of rotifer batch cultures grown with Culture Selco was characterised by continuous shifts in the dominant bands, whereas rotifers produced in a recirculation system had a more stable microbiota, which apparently evolved in relation to the quality and quantity of the rotifer cultures. *Marinomonas* and *Pseudoalteromonas* spp. were the prevailing members found (Rombaut *et al.*, 2001). A more complex microbiota was reported in similarly batch-grown rotifers enriched using Algamac 2000, where members of Rhodobacteraceae and *Arcobacter* sp. were dominant, but also including *Morganella morganii*, *Dyadobacter*, *Marinomonas*, *Roseobacter*, *Vibrio* and *Alteromonas* spp. (McIntosh *et al.*, 2008). The differences in the bacterial community demonstrated by recent studies applying 16S rRNA-based PCR-DGGE analysis may be related to the different culture and enrichment conditions used for rotifers. The bacterial community of a rotifer culture, grown on yeast and enriched with microalgae, was composed of Gram-negative bacteria belonging to alpha-Proteobacteria (*Ruegeria* spp.), gamma-Proteobacteria (*Marinobacter* sp. and an Oceanospirillaceae bacterium) and the Cytophaga-Flexibacter-Bacteroides group (*Polaribacter dokdonensis* and *Tenacibaculum soleae*) (Prol-Garcia *et al.*, 2010).

Artemia feeding generally succeeds to enriched rotifers few weeks ph. Unlike rotifers, each new production batch is obtained from rehydrated and disinfected cysts hatched into nauplii, then enriched into a metanauplii to increase its nutritional value. At this stage, culturable microbial load may reach levels as high as 10^{7-8} CFU mL⁻¹ in water and 10^{4-5} CFU per *Artemia* with a large proportion of bacteria belonging to the *Vibrio* group, mainly *V. alginolyticus* (Olsen *et al.*, 2000; Villamil *et al.*, 2003). Haemolytic bacteria have been detected in *Artemia* metanauplii, representing about 10% of the culturable microbiota (Olsen *et al.*, 2000). In fact, these authors suggested that the enrichment food given to *Artemia* might

influence the resulting microbiota. This coincides with the findings of Hoj *et al.* (2009) who observed a significant increase in culturable bacteria of enriched *Artemia*, but to an extent related to the enrichment product used (DC SuperSelco® <microalgae < DHA Protein Selco®) and resulting in different proportions of vibrios (0.1-1% for microalgae and DC SuperSelco® in contrast to 10% for DHA Protein Selco®). Using a combination of culture-based, microscopy-based and molecular methods, they analysed the localisation, abundance and community structure of bacteria associated with *Artemia* nauplii. Most bacteria were localised in the nauplii gut, with limited bacterial colonisation on external surfaces. The bacterial community of newly hatched nauplii was dominated by members of gamma-Proteobacteria and Planctomycetales. Enrichment with microalgae also led to the detection of *Antarctobacter* spp., a gamma-Proteobacterium (95% similarity to *Haliea rubra*) after use of both Selco products as well as *Vibrio* spp. for DHA Protein Selco®. The culture-independent microbiota of *Artemia* enriched with several products was found to differ from two production batches in successive years, dominating in *Vibrio pacinii* or in species of *Vibrio* and *Alteromonas*, and a Pseudoalteromonadaceae family member (McIntosh *et al.*, 2008).

Fish pathogens can be divided into two groups, as “primary” being the primary cause of disease or “secondary” being opportunists that invade already stressed or weakened larvae. The influence of pathogenic bacteria at the early cod larval stage was not demonstrated until recently. Sandlund and Bergh (2008) screened 53 potentially pathogenic bacteria in cod larvae using a multiwell system and found only four strains of *V. anguillarum* and one *Carnobacterium* sp. to cause mortality at a dose of 10^6 CFU mL⁻¹. A fifth strain of *V. anguillarum* as well as two other strains, *Aliivibrio logei* and *V. splendidus*, were found to be less virulent and required a longer incubation period to cause mortality. They were suggested to be secondary pathogens or that the administration mode was inadequate. The dominance of *A. logei* and *Vibrio xuii* in moribund cod fry was detected in two Norwegian hatcheries (Brunvold *et al.*, 2007), while *V. anguillarum* was identified from an unsuccessful larval tank (Reid *et al.*, 2009). Reid *et al.* (2009) tested potentially pathogenic isolates via rotifers (10^6 CFU per individual) fed to young cod larvae in a multiwell system and found that most of the *V. anguillarum* isolates (6/7), *V. splendidus* (1/3) and *A. logei* (1/1) were virulent. This is the first proof of pathogenicity for *A. logei* and *V. splendidus* DMC-1 in cod larvae but it still needs to be confirmed whether, as for *V. splendidus* (Gatesoupe *et al.*, 1999; Reid *et al.*, 2009; Thomson *et al.*, 2005), only certain *A. logei* strains from the rearing environment or moribund larvae are virulent. A recent attempt in elucidating the possible route of *V. anguillarum* infection in cod larvae could not confirm its port of entry (Engelsen *et al.* 2008). Reid *et al.*

(2009) demonstrated that *V. anguillarum* pathogenicity to cod larvae was influenced by the mode of administration, requiring a lesser concentration via rotifer feeding (10^6 CFU mL⁻¹) than via the rearing water (10^7 CFU mL⁻¹).

1.4 Other parameters influencing larval success

The term “larval success” suggests the development of healthy larvae within a reasonable timeframe and to an appreciable level that such larval rearing is considered sustainable and economically viable. The main determinants of larval success may be considered as intrinsic and extrinsic parameters which cannot necessarily be controlled. This is well demonstrated by the fact that variability in larval survival rate has been widely observed among sibling rearing units of apparently controlled hatcheries (Bjornsdottir *et al.*, 2009; Griffiths *et al.*, 2001; Nakase *et al.*, 2007; Shields *et al.*, 1999; Skjermo & Vadstein, 1999). As already discussed, suboptimal rearing conditions will inevitably have a negative effect, but the inherent status of ova or larvae should not be overlooked. Heredity, maternal influence, nutrition, biological and chemical control of rearing systems as well as good farming practices are key factors. Environmental stress renders larvae more susceptible to pathogens/opportunistic bacteria, having adverse effects on their viability. Bacterial abundance in larval rearing systems is not, in itself, a determinant of survival (Verner-Jeffreys *et al.*, 2004). In fact, quantitative microbiological evaluation of rearing systems alone has proven to be less informative than when conjugated to their qualitative analysis (Bjornsdottir *et al.*, 2009; Makridis *et al.*, 2000; Reid *et al.*, 2009; Ringo *et al.*, 1996; Ziaei-Nejad *et al.*, 2006). It can be realistic to estimate that about 70-80% of fertilised cod eggs will hatch successfully, while only 20% of the resulting larvae will reach the early juvenile stage (Steinarsson, 2004).

1.4.1 Ova and offspring quality

To date, it has not been possible to effectively predict gamete quality prior to fertilisation. Ova quality is therefore defined as the ability of the ova to be fertilised and to subsequently develop into a normal embryo (Bobe & Labbe, 2010). Recording of fertilisation rates is relatively easy for species with transparent eggs such as cod. Fertilisation rate and rates of normal blastomeres at early ova stages have been shown to be a useful predictive tool for evaluation of the potential juvenile production in turbot (Kjorsvik *et al.*, 2003). However, Penney *et al.* (2006b) reported the lack of a consistent relationship of morphological and

biochemical parameters with elapsed time in cod eggs produced by communally spawning broodstock fish. They pointed out that other more sensitive indicators of cod egg quality with higher predictive power of future viability are needed if operational efficiencies in egg selection and hatchery rearing are to be achieved.

The nutritional status of broodstock is certainly an important determinant of offspring quality (Blom & Dabrowski, 1996; Izquierdo *et al.*, 2001). Lipid and fatty acid composition of broodstock diet have been identified as major dietary factors that determine successful reproduction and survival of offspring, as reviewed by Izquierdo *et al.* (2001). Diet richness in essential fatty acids and interaction of ascorbate, carotenoids and vitamin E in reproductive fish physiology may influence the enhancement of the quality of gametes (Bobe & Labbe, 2010). The antioxidant function of ascorbate in teleost fish gametogenesis is critical to the fertilising ability of sperm and ova and more specifically the genetic integrity of gametes (Dabrowski & Ciereszko, 2001).

1.4.2 Rearing aids and feed at early stages

Inadequate feeding at early larval stage may lead to deprivation of essential nutrients with severe consequences such as larval malformation or death. Detailed understanding of the larval nutritional physiology and requirements has not yet been attained (Conceicao *et al.*, 2010). Rearing of marine fish implies the use of different components which all may influence its success. Microalgae are an important constituent of the oceanic food chain and they generally contain a high level of polyunsaturated fatty acids (PUFA). Use of microalgae during live feed enrichment or to shade the rearing water is a common practice in marine fish rearing. Several benefits have been reported: higher rotifer growth rate, reduction of the total heterotrophic microbial load and/or vibrio contamination, microbiota control, higher larval growth and survival, as well as better feed ingestion (Eddy & Jones, 2002; Makridis *et al.*, 2009; Nakase *et al.*, 2007; Oie *et al.*, 1997; Qi *et al.*, 2009; Reitan *et al.*, 1997; Rocha *et al.*, 2008). Microalgal production is complex and expensive, where up-scaling of the microalgal cultures takes place in several steps. During microalgal culture, a high organic load is progressively accumulated, which becomes a substrate for the proliferation of bacteria. Unsuccessful culture of microalgae, characterised by low growth rates and an extended lag phase, may result in a high bacterial load (Salvesen *et al.*, 2000). A fast-growing range of commercial products is available, including live microalgae concentrates, and frozen and freeze-dried microalgae.

Feeding of most species of interest for aquaculture still relies on live feeds during the early life stages. In the ocean, marine fish larvae obtain a large supply of nutrients by consuming plankton after first-feeding. Van der Meeren *et al.* (2008) compared the biochemical composition of copepods, zooplankton and live feeds and determined several differences. Total lipid concentration and lipid composition differed considerably between copepods and live feeds, importantly with greater arachidonic acid (ARA, C20:4n-6) levels and lower DHA and EPA levels in live feeds resulting in lower ratios of DHA/EPA (2-fold) and EPA/ARA (7-fold) than observed in copepods. Differences in protein-bound amino acids (PAA), FAA and pigment content were also observed. Copepods are nutritionally beneficial due to their naturally high levels of the essential highly unsaturated fatty acids (HUFA), namely EPA and DHA, which are predominantly in the form of phospholipids (Bell *et al.*, 2003). Fish oil emulsions can be used to enrich rotifers while *Artemia* are difficult to enrich with high levels of DHA and the HUFA tend to be located in triglycerides rather than phospholipids.

The digestibility and availability of HUFA supplied to fish larvae as pre-formed phospholipids may explain the superior efficacy of copepods and rotifers. Wold *et al.* (2007) demonstrated that DHA and EPA are more efficiently used in cod larvae when originating from phospholipids (PL) than neutral lipids (NL), resulting in increased growth associated with an earlier maturation of the intestine. The lipid source has also been shown to influence larval performance, with PL of marine source enhancing digestive functions compared to soybean lecithin which lacks HUFA (Cahu *et al.*, 2009). Since marine fish cannot synthesise HUFA *de novo*, they have been identified as essential dietary components (Cahu & Infante, 2001). The requirement for ARA and omega-6 docosapentaenoic acid (ω -6 DPA, C22:5n-6) should also be considered in the nutrition of cod. Garcia *et al.* (2008) reported that high concentration of DHA, EPA, ARA and ω -6 DPA in cod larvae following rotifer feeding led to better growth and survival while high larval DHA/EPA ratio ($\sim$ 3) and high DPA/ARA ratio (>1) following *Artemia* feeding could be linked to better survival. Park *et al.* (2006) found a DHA/EPA ratio in newly hatched cod larvae of 2:1, with increasing ratio as larvae grew. The significance of this increase was related to the DHA and EPA concentrations in rotifers. Though, the optimal level of dietary PL for cod larvae has not been established yet (Wold *et al.*, 2009).

Rotifers generally contain a high fraction of soluble protein (44.28–52.32%) with about 50% of total AA as essential AA, most of which were present at adequate levels related to general fish requirement (Srivastava *et al.*, 2006). Based on a study by Hamre *et al.*

(2008a), it is recommended to enrich rotifers used for marine fish larval culture with iodine and selenium as their use led to enhanced cod survival. Another study suggested the necessity to develop enrichment techniques to produce rotifers with sufficient amounts of all micronutrients corresponding to the levels found in copepods (Hamre *et al.*, 2008b). The FAA composition of live feed used in aquaculture may to some extent be manipulated within rearing conditions and species and strain selection. While microdiets are a promising feed for larval fish, no satisfactory techniques have at present been developed that allow the delivery of high contents of FAA at first-feeding.

1.4.3 Chemical indicators

Intensive rearing may lead to the accumulation of compounds toxic to larvae. This is more likely to occur in a recirculating than a flow-through rearing system. The assessment of chemical load at different stages in the aquacultural environment is important because it will reveal which parameters must be better controlled to maintain a desirable balance in the environment. The study of Björnsson and Ólafsdóttir (2006) indicated that rearing water quality had greater effects on the growth rate of cod juveniles (37 g), their nutritional condition and natural mortality than stocking density did. Earlier studies related to salmon rearing demonstrated that chemical parameters of importance include those constituting the organic load (chemical oxygen demand (COD), Kjeldahl nitrogen (TN), total phosphorus (TP), fatty materials) and chemicals toxic to living organisms (nitrite, hydrogen sulfide, metals and ammonia) (Auðunsson, 2002). Metals of interest are aluminium (Al), iron (Fe), zinc (Zn) and copper (Cu) especially, as its presence in small amounts has shown to influence severely the growth and survival of halibut (G.A. Auðunsson, oral communication). Threshold values for rearing water are 0.01 ppm (Al), 0.15 ppm (Fe), 0.005 ppm (Zn) and 0.03 ppm (Cu; or 0.1 ppm for alkaline water) (Timmons *et al.*, 2002). The toxicity of these metals is though reduced in presence of hard water (Howarth & Sprague, 1978).

The presence of organic material in the rearing water is undesirable since it may conduce to unwholesome bacterial proliferation. Excess feed and nutrient, nitrogenous wastes as well as water exchange rate and other rearing parameters may all influence the chemical load. Ammonia is the primary endproduct of nitrogen metabolism in teleost fish, but it can undergo bacterial degradation to nitrite (NO_2^-) and nitrate (NO_3^-). This reduces the toxic effects since aquatic organisms tolerate better nitrate (up to 500 ppm) than NH_3 or nitrite (toxic >1 ppm) (Moe, 1993). However, nitrification of NH_3 to nitrate depends on several

parameters, such as concentration of NH_3 , and dissolved oxygen, temperature, pH and presence of nitrifying bacteria (Hargreaves, 1998). High rearing water pH (>8.5) due to elevated NH_3 concentration (0.1-1 ppm) may inhibit nitrite oxidation, resulting in its accumulation (Belser, 1979). Ammonia toxicity depends primarily on ammonia (NH_3) concentration and environmental pH while in seawater NH_4^+ concentration may contribute to the toxicity as well (McKenzie *et al.*, 2003). Nitrite toxicity is, in turn, related to the concentration of nitrite and chloride in the water. Both ammonia and nitrite are stress factors, stimulating the release of corticosteroid hormones into the blood circulation where elevated concentrations impair the immune system and, consequently, decrease disease resistance. Ammonia and nitrite toxicosis is characterised by reduced survival and growth, and causes a variety of physiological dysfunctions (Tomasso, 1994). A negative linear relationship has been found between plasma ammonia concentration and maximum sustainable swimming speed in salmonids (McKenzie *et al.*, 2003).

Lower threshold values have been declared for ammonia toxicity in marine fish. Barnabè (1994) proposed the range 0-0.05 ppm to be safe while values above 0.1 ppm were generally harmful, but depending on fish species. This agrees with the findings of Alderson (1979) for turbot and El-Shafai *et al.* (2004) for tilapia (*Oreochromis niloticus*). Hydrogen sulfide is also very toxic to living organisms (Spotte, 1979), with a threshold value of 0.002 ppm in rearing water (Timmons *et al.*, 2002). Phosphorus concentration should range around 0.01-3 ppm while TN should be maintained below 1 ppm (Timmons *et al.*, 2002).

1.5 Current and novel prophylactic methods

The term “prophylactic” implies the ability to prevent the development of a disease. Current prophylactic methods in aquaculture may not totally achieve this aim. Antibiotics have been used to prevent and control bacterial diseases in aquaculture, but they can lead to the emergence of resistant bacteria and environmental problems (Serrano, 2005). It is therefore generally agreed that prophylactic antibiotics should be avoided or kept to a minimum. Following the development of immunoprophylactic control measures over the last two decades, the use of antibiotics in aquaculture has been reduced considerably (Gudmundsdottir & Bjornsdottir, 2007). Immunoprophylactic control of fish diseases involves classical vaccinology, pro- and prebiotic treatments as well as immunostimulation, all aiming to prime the innate and/or the adaptive immune system ahead of infection (Magnadottir, 2010).

Several fish diseases may affect cod, including parasitic, bacterial and viral infections. Vaccines based on inactivated bacterial pathogens have proven to be very efficacious in several fish species, while fewer viral vaccines are commercially available and no parasite vaccines exist (Sommerset *et al.*, 2005). Vaccination is though restricted to cod juveniles of at least 2-3 months old, as discussed earlier. Other prophylactic methods must be applied to enhance larval development and strengthen larval viability.

The microbial load in hatchery rearing may be reduced by water treatment and disinfection, but such treatments may disturb the natural balance in microbial communities and favour the proliferation of opportunistic bacteria. The same applies for the disinfection of live feed culture. This was shown by Hoj *et al.* (2009) who found *Vibrio* spp. to be a rather tolerant bacterial group compared to other members of the bacterial community in *Artemia* when treated with formalin, Virkon and an antibiotic. In order to avoid pathogen transmission in hatchery production, ova disinfection should be an integral part of the hygienic measures that should also include stringent control of water quality and other potential routes of disease transmission, such as feed, equipment and personnel. Alternative microbial control methods are needed to achieve a stable and successful production in hatcheries.

1.5.1 Ova disinfection

Since the surface of cod ova provides a good substratum for bacterial development (Hansen & Olafsen, 1989), application of a surface disinfectant may prove especially beneficial to their survival and hatching success. Several ova disinfectants have been evaluated, such as ozone (Grotmol *et al.*, 2003), iodophors, hydrogen peroxide and sodium hypochlorite (Bergh & Jelmert, 1996; Peck *et al.*, 2004). Use of glutaraldehyde, which has a rapid antimicrobial action and a broad spectrum activity (Gorman *et al.*, 1980), has been documented for ova disinfection of several marine fish, like sea bream, spotted wolffish (*Anarhichas minor*), Atlantic halibut, striped trumpeter (*Latris lineata*), turbot, plaice (*Pleuronectes platessa*) and Atlantic cod (Escaffre *et al.*, 2001; Hansen *et al.*, 2001; Harboe *et al.*, 1994; Morehead *et al.*, 2003; Salvesen *et al.*, 1997; Salvesen *et al.*, 1995). Salvesen and Vadstein (1995) compared the efficacy of glutaraldehyde in marine ova disinfection with that of other chemicals (chloramine T, iodine and sodium hypochlorite) and concluded that glutaraldehyde was the most promising agent. Salvesen *et al.* (1997) also found that glutaraldehyde disinfection led to reduced hatching time and more synchronous hatching as compared with untreated ova.

Therefore its use implies that newly hatched larvae may spend a shorter time in contact with the poor quality rearing water of the incubator before their transfer to a hatchery rearing unit.

Based on earlier literature, Escaffre *et al.* (2001) suggested the apparent inverse relationship between tolerance to glutaraldehyde and incubation temperature, but specified the need for intra-species testing. Indeed, the activity of glutaraldehyde is affected by temperature and pH (Gorman *et al.*, 1980). However, variation in the bactericidal effect of identical disinfection conditions has been shown to occur between different batches and to be correlated to the initial bacterial load of ova (Salvesen & Vadstein, 1995). Successful doses reported from the above mentioned studies are 400 ppm for 10 min at 5 °C, reducing time to 2.5 min at 12 °C, but using only 200 ppm for 4 min at 18 °C. In turn, Peck *et al.* (2004) investigated the effect of disinfection at different cod ova stages on the hatching success, which was found to depend on the type of disinfectants used. Earlier testing of glutaraldehyde on sea bream ova demonstrated that manipulation and treatment before the blastopore closure stage induced mortality (Escaffre *et al.*, 2001), explained by ova sensitivity to mechanical and chemical stress at early embryonic stage (Bergh & Jelmert, 1996).

More recently, Treasurer *et al.* (2005) reported that survival and hatchability of marine finfish ova could be improved by Pyceze[®] (50% (w/v) bronopol, 2-bromo-2-nitro-1,3-propanediol) disinfection, 50 ppm found to reduce 100-fold the microbial load on cod eggs after a 30-min static treatment *in situ*. Birkbeck *et al.* (2006) demonstrated *in vitro* the activity of bronopol against a range of marine organisms in seawater, with most bacteria having a minimum inhibitory concentration below 50 ppm. Difference in bacterial sensitivity was observed, being greater for *Vibrio* and *Moritella* spp. than for *Tenacibacter ovolyticus*, *Pseudomonas*, *Pseudoalteromonas*, *Colwellia*, *Photobacterium phosphoreum* and *Cytophaga/Flexibacter* spp.

1.5.2 Biocontrol and bioremediation

Biocontrol is a treatment antagonistic to pathogens and opportunistic bacteria, therefore aiming at their load reduction in the rearing water. It implies the use of natural components. Bioremediation involves the improvement of water quality through bioaugmentation by adding bacteria that reduce pollutants/waste in the rearing water and render it less stressful to the cultured organism. Nitrification may occur naturally in presence of *Nitrosomonas* and *Nitrobacter* spp. However, both species are slow-growing and may not become established due to water exchange in the rearing system. Thus, correct concentrations of bacteria on a

stabilised substrate must be implemented to achieve bioaugmentation (Panigrahi & Azad, 2007). By addition of *Bacillus subtilis* (Kernbio[®], Guangdong, China) and nutrients, bacterial assimilation was achieved, contributing to the removal of dissolved organic matter and total ammonia nitrogen in prawn (*Macrobrachium rosenbergii*) nursery wastewater which became a “microbially matured” water (Liu & Han, 2004). A recent study applied microbial mats collected from natural environments and constituting of bacteria, Cyanophyte, diatoms, nematods and Chlorophyte algae, to a recirculating system integrating shrimp (*Litopenaeus vannamei*) culture, resulting in a significant daily reduction of ammonia nitrogen, biochemical oxygen demand and suspended solids compared to the control systems as well as higher larval growth and survival (Lezama-Cervantes *et al.*, 2010). Recirculated water can lead to less variable rearing conditions, with a more stable larval survival and microbiota development (Verner-Jeffreys *et al.*, 2004). Recirculation systems require a biofilter that promotes recolonisation of the water by non-opportunistic bacteria, a process called microbial maturation which may result in increased larval survival and microbial control of rearing water (Skjermo *et al.*, 1997). Stabilisation of the microbiota is an ultimate aim. Other water treatments (filter, UV, chemicals or antibiotics) limit the microbial population and, by decreasing the level of microbial competition, may encourage the rapid proliferation of opportunistic bacteria in rearing water with high substrate availability.

1.5.3 Quorum quenching

A better understanding of bacterial pathogenesis may contribute to the development of novel prophylactic and therapeutic treatments. Bacteria ensure their survival and control their growth and metabolite production by different means. Quorum sensing (QS), a cell-to-cell communication and gene regulatory mechanism, allows bacteria to sense chemical signals (autoinducers) from other organisms in their surroundings and to form communities whose members interact with each other, either at the intra- or interspecies or even the interkingdoms level. Bacteria coordinate swarming, biofilm formation, stress resistance, production of toxins and metabolites by QS. Some pathogens make use of QS to control their virulence traits by assessing the local bacterial density and coordinating critical gene expression (Njoroge & Sperandio, 2009). QS using acylated homoserine lactones (AHLs) as signals regulates the expression of virulence factors in many Gram-negative bacteria (Waters & Bassler, 2005). Bruhn *et al.* (2005) reported the detection of AHLs among six of nine fish pathogenic species studied and Schwenteit *et al.* (2010) observed that AHL-mediated QS is involved in the

virulence regulation of *Aeromonas salmonicida* ssp. *achromogenes*. Interspecies communication has been suggested to occur via AHL signals in aquatic environments (Guan *et al.*, 2000). Interaction of different AHL molecules was shown to have a greater negative effect on the development and survival of prawn larvae than when applied singly, perhaps by instigating the production of various virulence factors in bacteria associated with the larvae (Baruah *et al.*, 2009). AHL regulation of bacterial virulence is likely to occur in intensive aquaculture systems.

Inhibition or inactivation of the signal molecules, called quorum quenching (QQ), has been shown to attenuate many of the processes controlled by QS. Different signal interference mechanisms against AHL QS systems have been unveiled: signal degradation by AHL degradation enzymes (lactonase, acylase and oxidoreductase), inhibition of signal biosynthesis by triclosan, and R protein degradation by halogenated furanones (Czajkowski & Jafra, 2009; Hoang & Schweizer, 1999; Manefield *et al.*, 2002). Interestingly, homologues of AHL-lactonase encoded by *aiiA* have been identified in a range of bacterial species, including *Bacillus thuringiensis*, *B. cereus*, *B. mycoides*, *Agrobacterium tumefaciens* and *Arthrobacter* sp. BLAST searches have indicated that this feature is common to many other bacterial species (Zhang, 2003). Finally, Tinh *et al.* (2008b) showed that adding AHL molecules to a larval turbot rearing system reduced survival, while the use of an AHL-degrading bacterial species could counteract this effect. Similar results were obtained for prawn larvae (Dang *et al.*, 2009). It is noteworthy that by quenching bacterial QS signaling and virulence factor production of pathogens, the host gains valuable time to activate defense mechanisms and to eliminate the invaders.

1.5.4 Probiotics

The demand for environmental friendly and sustainable aquaculture has led to increasing research on probiotics for aquatic animals. “Probiotics” traditionally refers to a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance (Fuller, 1989). Verschuere *et al.* (2000) introduced a new definition relating to the application of probiotics in the aquatic environment: a live microbial adjunct which benefits the host by modifying the host-associated or ambient microbial community, by enhancing the host response towards disease, by ensuring improved use of feed or enhancing its nutritional value or by improving the quality of its ambient environment. This suggests that

two vectors can be used for probiont transmission to larvae, either via the diet or the rearing water as proposed by Gatesoupe (1999).

In the search for probiotic candidates, the following criteria should be considered (Gatesoupe, 1999; Verschuere *et al.*, 2000): the microorganisms should (1) be non-pathogenic to the host, (2) compete with or hinder the growth of undesirable microbes, (3) adhere to, develop within the host, and (4) be indigenous to the environment to which it will be used. Many studies have demonstrated that the indigenous microbiota of fish or rearing environment can inhibit pathogen growth (Fjellheim *et al.*, 2007; Joborn *et al.*, 1997; Robertson *et al.*, 2000; Spanggaard *et al.*, 2001; Vijayan *et al.*, 2006; Vine *et al.*, 2004). Thus, a probiotic should have a proven safety and efficacy in the host (http://www.who.int/foodsafety/fs_management/en/probiotic_guidelines.pdf; 10.04.2008). Suggested modes of action resulting from probionts introduced in the GIT include the production of inhibitory compounds, competition with potential pathogens or opportunistic bacteria, inhibition of virulence gene expression, enhancing the immune response, improved gastric morphology and aiding digestive function. The application of probiotics may therefore result in elevated health status, improved disease resistance, growth performance, body composition, reduced malformations and improved gut morphology as well as microbial balance (Merrifield *et al.*, 2010).

Several probionts tested in fish aquaculture have been selected based on their effects in humans (Balcazar *et al.*, 2007; Nikoskelainen *et al.*, 2001; Panigrahi *et al.*, 2004) and livestock (El-Haroun *et al.*, 2006; Gatesoupe, 2002; Suzer *et al.*, 2008). These solutions may prove safe for human consumption but their efficiency in marine environment, as for coldwater fish, may be expected to be poor due to different requirements of non-marine, mesophilic microbes. Other researchers have focused their selection on autochthonous bacteria, i.e. those originating from the respective aquatic animals (Abd El-Rhman *et al.*, 2009; Aly *et al.*, 2008a; Bjornsdottir *et al.*, 2010; Brunt *et al.*, 2007; Caipang *et al.*, 2010a; Chabrillon *et al.*, 2006; Fjellheim *et al.*, 2010; Hjelm *et al.*, 2004; Irianto & Austin, 2002; Jatoba *et al.*, 2008). Overall, several positive characteristics have been demonstrated for probionts applied to various hosts, emphasising their potential in aquaculture production. These microbes generally include Vibrionaceae, pseudomonads, lactic acid bacteria, *Bacillus* spp. and yeasts. Questions have arisen as to whether dead or live microbial cells should be applied, at what concentration and frequency and finally through which vector (live feed or rearing water). Oral administration of live cells has proven to be more effective in pathogen-

challenged fry or modulation of immune response (Brunt & Austin, 2005; Panigrahi *et al.*, 2005; Taoka *et al.*, 2006).

Several approaches have been used to test candidate probiotics at different rearing stages. In larviculture of some marine species, probiotic supplementation has been successful via the rearing water (Makridis *et al.*, 2008; Ottesen & Olafsen, 2000; Plante *et al.*, 2007; Ringo & Vadstein, 1998; Strom & Ringo, 1993; Ziaei-Nejad *et al.*, 2006). This is relevant since the intestinal tract, skin and gills are generally accepted routes of entry for bacterial infection, such as vibriosis (Engelsen *et al.*, 2008; Ringo *et al.*, 2007). Live feed has also been an appropriate vector for probiont transmission to larvae (Carnevali *et al.*, 2004; Gatesoupe, 1991, 1994, 1997, 2002; Planas *et al.*, 2006; Plante *et al.*, 2007; Rollo *et al.*, 2006; Ziaei-Nejad *et al.*, 2006). Some of these findings have suggested the role of the gastrointestinal (GI) microbiota in the nutrition and health of fish by enhancing its growth, digestion, immune responses and disease resistance. However, at early larval stage of marine species such as Atlantic cod, the GI tract is poorly developed. The microbiota established may therefore reflect that of the environment and live feed which often carries high bacterial load (Olafsen, 2001). Hence, probiotics should be administered early and frequently for maximum effect. The possible benefit of probiotic treatment of ova has been evaluated in Atlantic cod (Hansen & Olafsen, 1989) and halibut (Ottesen & Olafsen, 2000) rearing, leading to increased larval survival in the latter study.

Research on the effect of probiotics administered to juveniles via feeding was initiated more than a decade ago and aimed mainly at assessing their disease reduction effect. Trials with cod juveniles, given feed supplemented with *Carnobacterium divergens* for three weeks, demonstrated that some protection was achieved upon challenging with *V. anguillarum* and that *C. divergens* was among the intestinal microbiota of surviving fish (Gildberg *et al.*, 1997). A similar experiment also showed a significantly reduced cumulative mortality 12 days post-challenge while the effect was lost 4 weeks post-infection (Gildberg & Mikkelsen, 1998). Other lactic acid bacteria (LAB) strains applied to different fish species led to increased survival compared with control groups when challenged with fish pathogens, as in trout (Irianto & Austin, 2002; Nikoskelainen *et al.*, 2001; Vendrell *et al.*, 2008) and tilapia (Aly *et al.*, 2008b). Disease resistance has been observed in presence of other Gram-positive and -negative bacterial species (Brunt & Austin, 2005; Irianto & Austin, 2002) along with increased growth (Ghosh *et al.*, 2008; Rengpipat *et al.*, 2003). Immune enhancement has been reported in trout (Brunt & Austin, 2005; Nikoskelainen *et al.*, 2001; Panigrahi *et al.*, 2004) and sea bream (Salinas *et al.*, 2005) given LAB-supplemented feed. Moreover, LAB modified

Table 2. Some commercially available “probiotic” products for aquaculture, their application purpose, benefits and microbial content

Product (company)	Application	Benefits mentioned	Microbial content	Ref
Sanlife® MIC-F (INVE)	water addition; fresh & marine cultures	produces enzymes; degrades waste products; improves water quality in fish hatcheries and live food production; optimises gut microflora	superior microbial mixture (<i>Bacillus</i> spp.)	1 ★
TOARAZE (TOA Pharmaceutical Company of Japan)	mixed to feed for eel and shrimp	inhibits GIT pathogens; improves feed effectiveness, growth rate and water quality; increases farming yield	<i>Enterococcus faecalis</i> T-110, <i>Clostridium butyricum</i> TO-A, <i>Bacillus mesentericus</i> TO-A	2
Bacta-Pur® N3000 (IET-Aquaresearch)	lobster holding tank	improves water quality; controls ammonia and nitrite	nitrifying and denitrifying bacteria	3
AQUA PROTECH (GRM Ltd.)	mixed to feed for prawn, shrimp and fish	ensures healthy gut microbiota; improves feed utilisation and FCR; enhances immune response; reduces disease incidence and mortality	<i>Saccharomyces cerevisiae</i> and several other components (herbal mineral complex, spirulina, seaweed, enzymes and vitamins)	4
Prowaso (Som Phytopharma India Ltd., Bio Ops Div)	soil and water probiotic		<i>Rhodococcus</i> , <i>Rhodobacter</i> , <i>Nitrosomonas</i> , <i>Nitrobacter</i> , several <i>Aspergillus</i> and <i>Bacillus</i> spp., <i>Pseudomonas putida</i> , <i>Trichoderma reesei</i>	5 ★
Progut (Bio Ops)	gut probiotic		several lactic acid bacteria, <i>Bacillus</i> and <i>Aspergillus</i> spp., <i>T. reesei</i>	5 ★
Biozeo (Bio Ops)	water addition	zeolite controller	microbes producing organic acid	5
Vibrichek	water addition	<i>Vibrio</i> controller	mainly <i>Bacillus</i> spp.	5 ★
Fungistat (Bio Ops)	water addition	fungistatic	<i>T. viride</i> , <i>T. harzianum</i> , <i>Ps. fluorescens</i> , <i>Ps. putida</i>	5
AQUAPRO-B	water addition	degrades organic material; reduces ammonia; prevents <i>Vibrio</i> growth and colonisation of surfaces and water	mainly <i>Bacillus</i> spp.	6 ★
Pro-3000 series (Aqua-in-tech)	water or feed	reduces stress and improves survival rates	<i>B. subtilis</i> , <i>B. licheniformis</i> , <i>Lactobacillus acidophilus</i> and <i>S. cerevisiae</i>	
ALKEN CLEAR-FLO® 1006 (Alken Murray)	shrimp, lobster, Koi prawn, salmon, halibut	improves digestion and feed conversion; enhances immune response and water quality; eliminates pollutants; reduces water turbidity	six Gram-positive spore-forming <i>Bacillus</i> spp. and ten Gram-negative bacteria	7 ★
BACTOCELL® (Lallemand)	feed additive for salmonids, shrimp	enhances salmonids and shrimp production	<i>Pediococcus acidilactici</i> CNCM MA18/5M	8
Several probiotic strains/products (Unique Biotech Limited)	water or feed addition	several properties	<i>Bacillus</i> and <i>Aspergillus</i> spp., <i>Lact. sporogenes</i> , <i>Rhodococcus</i> , <i>Nitrosomonas</i> , <i>Nitrobacter</i> , <i>Cellulomonas cartae</i> , <i>Ps. putida</i> , <i>Ps. denitrificans</i> , <i>Alcaligenes faecalis</i> , <i>S. cerevisiae</i>	9 ★

Biogen® (China Way Corporation)	feed addition	growth promoter	Allicin (≥ 0.247 Mmol g ⁻¹), <i>B. subtilis</i> Natto ($\geq 6 \times 10^7$ g ⁻¹), High Unit Hydrolytic Enzyme (≥ 3690 U g ⁻¹)	10
Alchem Poseidon (Alchem Korea)	water or feed addition	enhances non-specific immune parameters	<i>B. subtilis</i> ($> 1.6 \times 10^7$ CFU g ⁻¹), <i>Lact. acidophilus</i> ($> 1.2 \times 10^8$ CFU g ⁻¹), <i>Cl. butyricum</i> ($> 2.0 \times 10^7$ CFU g ⁻¹) and <i>S. cerevisiae</i> ($> 1.6 \times 10^7$ CFU g ⁻¹)	11
Lymnozyme® (Keeton Industries)	water addition	improves water conditions in shrimp and fish hatcheries by competitive exclusion of pathogenic microbes and reduction of mortalities; increases survivability and growth resulting in higher profits; improves <i>Artemia</i> survival by improving water quality for culture; effective in fresh, brackish or salt water applications, in a wide range of water temperatures and over a high pH range	natural microbial product (Patent No 6,878,373,B2)	12 ★
Efinol® PT	microbial culture for shrimp and fish ponds; feed coating	lowers stress effects; minimises mortality during disease outbreak; improves uniformity and yield of aquatic species; helps to maintain favorable microbial balance and water quality conditions in ponds	<i>B. subtilis</i> , <i>B. licheniformis</i> , <i>B. coagulans</i> , <i>Lact. acidophilus</i> , <i>Streptococcus faecium</i> , <i>S. cerevisiae</i>	13
Efinol® L (Bentoli, Inc.)	water addition	reduces stress; improves survival rates; produce stronger and healthier larvae and post-larvae	<i>B. subtilis</i> , <i>B. licheniformis</i> , <i>Lact. acidophilus</i> , <i>S. cerevisiae</i>	★
Kernbio® (Guangdong)	water addition	removes dissolved organic matter and total ammonia nitrogen by microbial remediation	10 ¹¹ spores of <i>B. subtilis</i> per tablet	14
Remus® (Avecom)	live feed or water addition	improves the quality and health of aquaculture systems; beneficial in larval and live food cultures	<i>Aeromonas schubertii</i> , <i>Paracoccus denitrificans</i> , <i>Phenyllobacterium</i> and <i>Gluconobacter</i> spp.	15 ★

★ indicates the possible suitability for cod rearing at early stages. All website information retrieved 11.02.2010 (unless otherwise stated):

- 1: <http://www.inve.com/INVE-Aquaculture/English/Products/Fish-hatcheries/Health-products/Sanoflife+MIC-F/page.aspx/1087>; (Decamp *et al.*, 2008)
- 2: <http://bio-three.com/aqua.php> 3: <http://www.bactapur.com/en/aquaculture-holding-tanks.htm> 4: <http://www.grmorg.com/grmaquaaculture.htm>
- 5: http://www.somphyto.com/probiotics_proaqua.htm 6: <http://aqua-in-tech.com/probiotics%20cover.htm> 7: <http://www.alken-murray.com/1006pib.htm>
- 8: http://www.lallemand.com/Home/eng/PDFs/LAN09-PR09-Bactocell_aqua_EN.pdf; (Gatesoupe, 2002) 9: <http://www.uniquebiotech.com>
- 10: Tested in Nile tilapia, *Oreochromis niloticus* L., fingerlings (El-Haroun *et al.*, 2006)
- 11: Probiotics introduced by feeding (live or dead cells) or supplying live cells to the tilapia rearing water in a closed recirculating system (Taoka *et al.*, 2006)
- 12: http://www.ecosmarte.com.au/waste_water/microbes.pdf
- 13: <http://bentoli.biz.efeedlink.com/Products/s6110-9cd37497-2d68-4b91-a384-767da3704658.html>; (Comes *et al.*, 2008; Gomes *et al.*, 2009)
- 14: Reuse strategy of wastewater in prawn nursery by microbial remediation (Liu & Han, 2004)
- 15: <http://www.tmc-ltd.co.uk/commercial/abil-remus.asp>; retrieved 25.10.2009; (Sveinsdottir *et al.*, 2009).

the gut microbiota of trout (Panigrahi *et al.*, 2004), while *Bacillus* strains achieved a microbiota effect in shrimp (Moriarty, 1998) and ornamental fish species (Ghosh *et al.*, 2008). Recently, a commercial probiotic product, Biogen[®], was shown to increase growth performance in tilapia (El-Haroun *et al.*, 2006). Further, probiotic feed has led to increased digestibility in shrimp (Lin *et al.*, 2004) as well as to increased enzymatic activity in abalone (*Haliotis midae*) (Macey & Coyne, 2006).

The effects of probiotics to fish larvae can be determined in several ways. *In vitro* testing of larvae in multiwell plates will restrict rearing variables and allow for a simple evaluation of survival and threshold probiont concentration needed. Putative immunomodulatory capabilities have also been determined under *in vitro* conditions (Caipang *et al.*, 2010a). *In vivo* testing in rearing units will enable a thorough assessment under normal rearing conditions, which may influence the outcome. However, larval size and disparity in rearing units may render sampling and analysis more difficult early posthatch. Still, much of the research on probiont efficacy in hatcheries has been based on quantitative and qualitative microbiota analysis, detection of probionts post-treatment, larval growth, development, viability and survival, as well as immune modulation, enzymatic activity in larvae and pathogen challenge studies. Lately, molecular approaches have extended the evaluation to the gene expression targeting factors related to the immune response, stress response (glucocorticoids/cortisol stress hormone and heat shock proteins levels), growth (insulin-like growth factor I and myostatin), welfare and metabolism (Avella *et al.*, 2010; Carnevali *et al.*, 2006; Reyes-Becerril *et al.*, 2008; Rollo *et al.*, 2006).

Table 2 lists several commercial products claiming to have probiotic properties in the field of aquaculture. The effects of only few of them have been demonstrated by *in vivo* studies published scientifically, as indicated by references given at the table foot. Some microbial strains are apparently common to certain products, especially *Bacillus* spp., *S. cerevisiae* and few LAB. Only a few products may be efficient at lower temperature and could therefore be suitable at early stages of cod rearing, i.e. by addition to the rearing water, or for live feed culture. However, the need for tailor-made probiotic solutions for successful larviculture has been suggested (Azad & Ai-Marzouk, 2008). The current study focused on the search of cod probiotics from the healthy rearing environment to suit the requirements at early stages. Similar research work has been under development for several other aquatic species. Future studies should emphasise practical industrial scale applications and demonstrate long term effects. Further, the production feasibility and cost-benefit analysis of developed probiotics should be approached.

2 AIMS OF THE STUDY

The overall aim of the study was to increase survival and promote development at early stages of cod rearing. A first step towards this goal was to improve knowledge of microbiological parameters of cod aquaculture, from postfertilisation through the larval period where mortality is high. Cultivation methods were used to retrieve bacterial isolates, allowing for their enumeration and characterisation, which could not be achieved by culture-independent methods. A second step was to identify and characterise probiotic bacteria from healthy cod aquacultural environment. Finally, the development of probiotic methods to control the microbiota and to improve cod performance was attempted.

This was achieved through the following specific objectives:

- To study the microbiota of cod rearing systems at early stages and define putative probiotic bacteria based on several *in vitro* tests (***Papers I and III***)
- To develop microbial treatments using putative probionts (***Papers II and III***)
- To assess the effects of microbial treatments at the earliest stages of cod rearing on the microbial load, larval growth and performance (***Paper II***)
- To compare the microbiota of treated and untreated ova and larvae (***Paper III***)
- To define positive and negative parameters influencing cod survival and development (***Papers II, III, IV and V***)
- To validate the use of two selected prospective probionts, *Arthrobacter* sp. and *Enterococcus* sp., at early stages of cod rearing by bathing ova and larvae, or feeding juveniles with mixed or single probiont diets (***Papers IV and V***)

3 MATERIALS AND METHODS

3.1 Cod rearing and experimental set-up

3.1.1 Fertilisation, disinfection and incubation of ova

Different experiments were conducted at the Aquaculture Research Station of the Marine Research Institute (Grindavik, Iceland). Spawning of wild cod occurred in late April 2004 and 2006, and mid-May 2005 off the West coast of Iceland. Fertilised cod ova were obtained at sea, brought to the rearing station (0 dpf) and stored in seawater under chilled conditions before disinfection. Ova were surface-disinfected with glutaraldehyde (300 ppm, 5 min), rinsed in seawater and transferred to 25-L incubators (7-8 °C). Seawater flow was adjusted to 0.5 L min⁻¹ and later on increased to 1 L min⁻¹ (*Papers I-II-III*). In 2004, ova were disinfected again 11 dpf.

In 2006 (*Paper IV*), fertilised ova were surface-disinfected with 50 ppm (0.1 mL L⁻¹) Pyceze[®] [50% (w/v) bronopol, 2-bromo-2-nitro-1,3-propanediol, Novartis Animal Vaccines, UK] for 30 min, rinsed in seawater and transferred to 25-L incubators (7-8 °C). On day 10 pf, 50 ppm Pyceze[®] was added to the incubators, intermitting seawater flow for 30 min.

3.1.2 Hatching and rearing of larvae

Upon hatching completion (0 dph), larvae were transferred to 150-L experimental silos or 3400-L tanks. Table 3 presents the larval rearing conditions for the three spawning seasons studied, indicating some of the differences between trials. In 2004, current hatchery practices were mainly examined to allow for the isolation and identification of cod rearing microbiota (*Paper III*), as well as the characterisation of putative probionts (*Paper I*). Probiotic treatments were developed and tested in experimental silos in 2005, evaluating their effects on the microbiota, larval performance and development (*Papers II and III*), and the two promising probionts validated in larval hatchery in 2006 and compared with Remus[®] (Avecom, Belgium), a commercial probiotic product (*Paper IV*).

Table 3. Larval rearing conditions and feeding regimes during the three spawning seasons studied

Spawning year	2004		2005		2006	
Postfertilisation period (days) ^a	13		12		13	
Posthatch period (days) ^b	15		27		36	
Rearing system	150 L		3400 L		150 L	
Treatment	none		antibiotics ^g			
Replicate	2		2		4	
Larval density (larvae L ⁻¹)	40		60		60	
Rearing water temp. (°C)	8-10		8-10		8	
Seawater flow (L min ⁻¹)	0.5		0.5		0.5-1.5 (20 dph)	
Lighting (lx 24 h day ⁻¹)	300		300		300-700 (5 dph)	
Algal concentrate (day ⁻¹) ^c	2 x 2 mL		2 x 2 mL		2 x 2 mL	
Rotifers (10 ⁵ individuals day ⁻¹) ^d	20 (3-14 dph)		20 (3-26 dph)		3 x (6-25) (3-30 dph)	
<i>Artemia</i>					nauplii: 2-5 (21-29 dph)	
(10 ⁵ individuals day ⁻¹) ^e					enriched: 4-10 (30-35 dph)	
Dry feed (max ration day ⁻¹) ^f						

^a Time from ova fertilisation to end of hatching in incubators; ^b Length of larval period studied; ^c Instant Algae® Nannochloropsis (Reed Mariculture, USA) added twice daily; ^d *Brachionus plicatilis* enriched with Protein Selco Plus (INVE, Belgium) given twice or three times daily; ^e *Artemia salina* (INVE), 24-h nauplii or 48-h metanauplii enriched with Gadi Selco (INVE); ^f Gemma Micro and Gemma products (Skretting, Norway); ^g Neomycin treatment (25 ppm, 1 h) applied 35 dph to both tanks and Interspectin-L WS (40 ppm, 1 h) added 55 dph to tank 1; Larvae bathed regularly with a bacterial mixture (04-279, 04-394, and CdV41 only in 2005); ^h Rotifers enriched in presence of 2 bacterial strains (04-342 and 04-683) and a probiotic yeast (Levucell SB20, Lallemand, France), given un-rinsed for the morning ration; ⁱ Enriched rotifers supplemented with a commercial probiotic (Remus®, 10 g morning ration⁻¹); S1 means silo 1, etc; dph, days posthatch.

3.1.3 Evaluation of pre-treatments of rotifer culture

The effect of Pyceze[®] concentration on the microbiological quality of rotifer culture after a 30-min treatment was evaluated. Rotifers were rinsed with seawater and allocated to 6 aerated seawater beakers to reach a final volume of 1000 mL and a density of 1400-1500 individuals/mL. Pyceze[®] was applied at 3 concentrations (25-50-100 ppm) in duplicate beakers. The culturable microbiota of the rotifer culture was evaluated quantitatively in duplicate samples at time 0 (no pyceze added) and after a 30-min treatment. Total viable count (TVC) was obtained using marine agar 2216 (MA, Difco, BD, USA) and presumptive *Vibrio* counts using thiosulfate-citrate-bile-sucrose agar (TCBS; Difco; dissolved in ASW) after incubation at 22 °C (3-4 days). Density of rotifers was evaluated at the end of the experiment.

Secondly, the effect of rinsing the rotifers (control) and optionally disinfecting with Pyceze[®] (75 ppm) for 30 min before enrichment on the microbiological quality of rotifer culture post-enrichment was evaluated. Further, the use of Remus[®] (100 ppm) during rotifer enrichment (12 h) was assessed. Remus[®] is a commercial probiotic product containing *Aeromonas schubertii*, *Paracoccus denitrificans*, *Phenylobacterium* sp. and *Gluconobacter* sp., originating from successful rotifer and *Artemia* cultures, and can improve the quality and health of aquaculture systems, being beneficial in larval and live food cultures (retrieved 25.10.09; <http://www.tmc-ltd.co.uk/commercial/abil-remus.asp>). After the completion of the two pre-treatments, rotifers were allocated to 4 aerated seawater beakers for each pre-treatment to reach a final volume of 1000 mL and a density of 1400-1500 individuals/mL. Then each group of pre-treated rotifers received either 0 ppm or 100 ppm Remus[®] in addition to the regular enrichment product (Protein Selco Plus). After 12 h, samples were then aseptically taken from duplicate treatments and analysed for TVC and presumptive *Vibrio* counts. The interaction between the use of Pyceze[®] and Remus[®] was statistically evaluated.

3.1.4 Pre-testing of putative probionts for rotifer culture

Rotifer culture takes place at 25-27 °C under an alkaline (7.5-8.5) and saline (2.0-2.5% NaCl) environment. Pre-testing of the putative probionts intended to be used in rotifer culture was performed *in vivo* at small scale in 2005 over a 74-h period to assess their performance in the system. Two bacterial strains (04-342 isolated from an algal concentrate and 04-683 from dry feed, see Table 4) were evaluated at an initial concentration of 10^{4-5} CFU mL⁻¹ as well as two commercial probiotic products, Remus[®] (0.1 g L⁻¹ as a recommended dose by the supplier)

and Levucell SB20[®] (a whole-cell *Saccharomyces cerevisiae* preparation applied as 10⁵⁻⁶ CFU mL⁻¹). The bacterial strains were previously grown at 22 °C for 40 h in tryptic soy broth (Difco) supplemented with 6 g L⁻¹ yeast extract (Difco) and 27 g L⁻¹ sea salts (Sigma-Aldrich, USA) (pH adjusted to 7.5, MYE-TSB) before being 10-fold diluted and added to the rotifer cultures. Rotifers were first antibiotically treated (50 ppm neomycin for 2 h) to reduce the microbial load and then allocated to 12 aerated seawater beakers to reach a final volume of 800 mL and a density of 500 individuals mL⁻¹. In total, 6 treatments were evaluated in duplicate: control, Remus, 04-683, L04-683, 04-342, and L04-342. Microbial supplements were added at time 0, except for Levucell SB20[®] (L) which was added after 3, 24, 48 and 69 h. Based on current practice, rotifer cultures were supplemented with Culture Selco Plus (INVE) over a period of 68 h and then enriched using Protein Selco Plus. The effect of each treatment on the rotifers' microbial load was evaluated after -2, 0, 3, 22, 68, 74 h using MA for TVC, TCBS (for presumptive *Vibrio* counts, Nitrite Actidione Polymyxin agar (NAP) according to Davidson and Cronin (1973) for presumptive lactic acid bacteria (LAB) counts and Dichloran Rose Bengal Chloramphenicol agar (DRBC, Difco) for yeast counts. All media were incubated at 22 °C for 3-4 days. LAB colonies were confirmed by catalase test using 3% H₂O₂.

3.1.5 Microbial treatments at larval stage

Five bacterial strains were used for microbial treatments of ova, larvae or rotifers, as listed in Table 4. Three bacterial strains [*Arthrobacter bergerei* (04-279), *Enterococcus thailandicus* (04-394) and *Carnobacterium divergens* V41 (CdV41)] were used for bathing ova and larvae in 2005 (**Papers II and III**) but only two of them (04-279 and 04-394) were applied in 2006 (**Paper IV**). Pre-culturing and bacterial suspensions were prepared daily prior to use as described in **Paper II**. Concentration of bacterial suspensions was 10⁹⁻¹⁰ CFU mL⁻¹ for strains 04-394 and CdV41, but 10-fold higher for 04-279. Ova were bathed few times with bacterial strains (10⁶⁻⁷ CFU mL⁻¹ rearing water for 30 min with intermitted flow) during the incubation period, while larval bathing was performed regularly in the silos by adding each bacterial suspension (ca 10⁶ CFU mL⁻¹ rearing water) as described in **Papers II, III and IV**.

Treated rotifers (*Brachionus plicatilis*; 10⁶ individuals L⁻¹) were inoculated upon enrichment (Protein Selco Plus) with 50 mg Levucell SB20 and 10 ml culture of two bacterial strains (04-342 and 04-683, Table 4) grown at 25 °C (48 h) in MYE-TSB. Initial cell concentration of added bacteria and yeasts was 10⁶⁻⁷ CFU mL⁻¹ rotifer culture. Treated rotifers

were given un-rinsed (until 20 dph) to each respective silo (S5-S6) for the morning ration (**Paper III**).

Remus-rotifers were prepared for the morning ration after enrichment by adding a commercial probiotic product, Remus[®] (10 g ration⁻¹), for 30 min before being given un-rinsed to each respective silo, S9-S12 (**Paper IV**).

Table 4. List of bacterial strains isolated from the cod hatchery and salmon intestine, and applied for treatments at early stages of cod rearing

Rearing application	Strain code	Closest match in GenBank (% similarity)	Accession number	OTU code ^a	Strain origin
Bathing of ova and larvae	04-279	<i>Arthrobacter bergerei</i> (100)	AJ609631	Ac-C2	cod larval rearing water ^b
	04-394	<i>Enterococcus thailandicus</i> (99)	EF197994	Fi-C1	algal concentrate ^b
	CdV41	<i>Carnobacterium divergens</i> (100)	AY543036	-	salmon intestine ^c
Treated rotifers	04-342	<i>Alkalibacterium olivoapovliticus</i> (99)	AF143511	Fi-C7	algal concentrate ^b
	04-683	<i>Enterococcus faecium</i> (98)	AF039901	-	dry feed ^b

^a OTU (operational taxonomic unit) code defined for sequenced isolates identified more than once; ^b **Paper I**; ^c Pilet *et al.* (1995).

3.1.6 Preparation of probiotic feeds for juvenile trials

Two probionts (strains 04-279 and 04-394) were selected for juvenile feeding trials. Expanded dry feed (1.6 mm MINI 22/50, Fódurblandan hf, Iceland) was coated with 1% (v/w) of each bacterial suspension and air-dried in a sterile stainless steel tray at 50 rpm in a laminar flow cabin at room temperature for 2 h. First, their viability and stability on coated feed was evaluated in vacuum-packed portions (20 g) stored for 8 weeks at -20, 4 and 15 °C as described in **Paper V**. Triplicate samples were evaluated at packaging and every two weeks. Feed preparation for the juvenile trials was done as described above, 2 kg of each feed type vacuum-packed in 250 g portions in plastic bags (PA55/LDPE60). Four different feed types were prepared, one control feed and three treated with probiont suspensions. A-feed contained both probionts, B-feed only strain 04-279 and C-feed only strain 04-394. At the aquaculture station, the feed bags were stored chilled until used (**Paper V**).

3.1.7 Juvenile rearing

Cod juveniles (10 ± 1 g), obtained 5 months posthatch from 5000-L tank (10°C), were transferred to 12 flow-through ($1\text{ L kg}^{-1}\text{ min}^{-1}$) aerated 300-L tanks ($80\text{ juveniles tank}^{-1}$) for acclimatisation, 2 days before the feeding experiment started. Seawater temperature was $7.5 \pm 0.2^{\circ}\text{C}$. Dry feed was rationed to each tank, but not given to juveniles the evening preceding the morning sampling. Dead fish were removed daily and recorded. At the end of the feeding trials, five juveniles were randomly removed from each tank, anaesthetised [tricaine methane sulphate (TMS, Syndel Laboratories, Canada), 50 mg L^{-1} seawater], weighed, and their length recorded. Three of the juveniles from each group were used for microbiological analysis.

3.1.8 Experimental set-up

All cod trials were carried out according to the Icelandic Animal Research Authority (approval no. YDL03080041/023BE). A schematic presentation of the three larval trials performed during the 2004-2006 spawning seasons of wild cod and of the juvenile probiotic intervention study conducted in the fall of 2006 is shown in Figure 3, including the treatments and the sampling scheme.

In 2004, ova and larval rearing units were studied early postfertilisation through hatching until weaning (56 dph), including all feeding diets (*Papers I and III*). Two microbial treatments, bacterial bathing of ova and larvae or larval feeding with microbially treated rotifers were compared in duplicate with untreated rearing systems in 2005 (*Papers II and III*). Finally in 2006, two probiont validation trials were conducted. First at larval stage, a 2-strain bacterial mixture was applied via the rearing water late postfertilisation and the following 4 weeks posthatch with four replicates, being compared with four untreated controls and four silos receiving rotifers supplemented once daily with a commercial probiotic, Remus[®] (*Paper IV*). Secondly, an intervention study using probiotic feeds was performed at early juvenile stage to confirm the properties of two prospective probionts (*Paper V*). This last experiment was divided into two subtrials: (I) 6 tanks given control or A-feed for 28 days (3 replicates each), and (II) 6 tanks administered control, B or C-feed (2 replicates each) for 55 days.

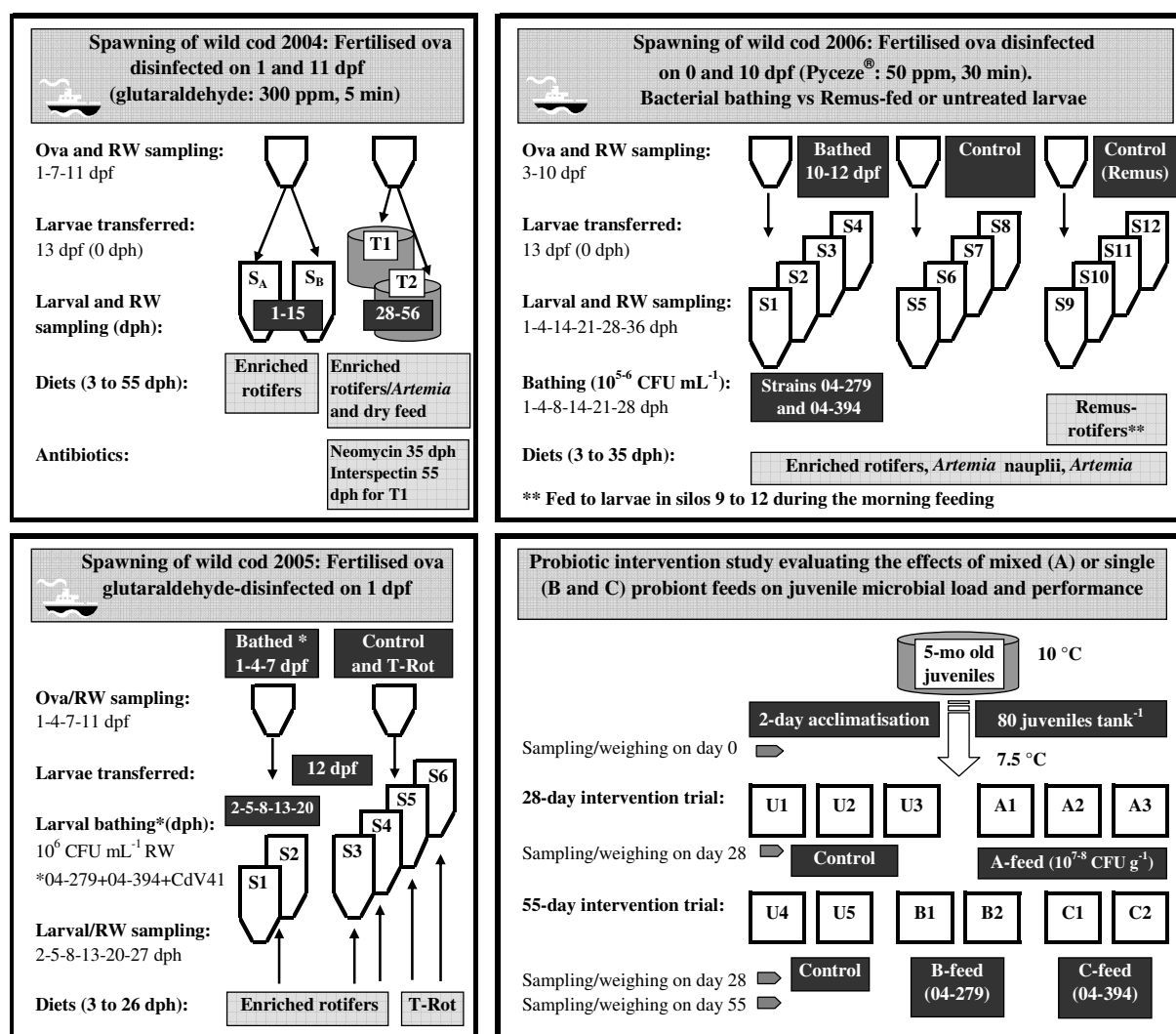


Figure 3. Experimental treatments and sampling scheme for cod rearing systems evaluated at larval and juvenile stages

RW, rearing water; dpf, days postfertilised; dph, days posthatch; S_A, silo A; S1, silo 1, etc.; T1, tank 1, etc.; CFU, colony-forming unit; T-Rot, rotifers enriched for 12 h in presence of 2 bacterial strains (04-342 and 04-683) and a probiotic yeast (Levucell SB20), given un-rinsed for the morning ration.

3.2 Sampling and analysis of rearing systems

3.2.1 Sampling of cod rearing systems

Sampling performed for probiont isolation and microbiota analysis of rearing systems in 2004 and 2005 took place during the pre- and posthatch periods (*Paper I-II-III*). Samples analysed included incoming seawater, ova, larvae (mainly GIT), rearing water, algal concentrate, rotifers, *Artemia* and dry feed. Juveniles (5-6 months) were sampled from three tanks during a visit in October 2004 for the search of probionts. In the spring of 2006, samples of rearing water, ova, larvae and rotifers were obtained (*Paper IV*). In the fall of 2006, samples of

rearing water, feeds, juvenile gut and gills were analysed (*Paper V*). Table 5 summarises all the samples analysed. Figure 3 presents the sampling scheme.

Table 5. List of samples analysed and associated with larval and juvenile rearing systems

	<i>Paper I</i>	<i>Paper II</i>	<i>Paper III</i>	<i>Paper IV</i>	<i>Paper V</i>
	2004	2005	2004-2005	2006	2006
Seawater	-	1	-	1	-
Rearing water (ova stage)	4	10	4	1	7
Ova	5	6	5	4	6
Rearing water (larval stage)	7	28	7	2	62
Larvae (newly hatched)	2	2	1	2	-
Larvae (surface-disinfected)	6	11	6	5	24
Algal concentrate	2	1	2	-	1
Rotifer culture	3	6	3	2	6
Artemia culture	1	-	1	2	2
Dry feed	1	-	1	-	-
Juvenile gills	3	-	-	-	-
Juvenile gut	-	-	-	-	-
Rearing water (juvenile stage)	-	-	-	-	-
Total number of samples	34	65	30	19	108
					80

Samples of larvae and rearing water were obtained early morning before supplement addition. All samples were aseptically prepared, diluted in cooled (3-5 °C) diluent [ASWP, 50% artificial seawater containing 20 g L⁻¹ sea salts (ASW) and 0.1% (w/v) peptone, Difco] and analysed as described in *Paper I*. Fertilised ova were rinsed (cooled ASW) before analysis to remove non-adherent microorganisms. Larvae were anaesthetised [0.17% (w/v) hypnodyl for 30 s], surface-disinfected [0.1% (v/v) benzalkonium chloride solution] and rinsed three times. This allowed for the isolation of larval GI microbiota. A pool of whole larvae (40-130 individuals depending on age) was homogenized, diluted and mixed. In 2004, rotifer and *Artemia* cultures were sampled after enrichment and stored chilled until analysed. In 2005, rotifer cultures were sampled at different production steps (during and after enrichment, and after rinsing). The live feed samples were then vortexed for 30 s and diluted as required. Dry feed and algal concentrate were weighed, diluted 10-fold, mixed in a

stomacher (Seward Stomacher 400; Lab Systems, Seward Ltd, UK) for 60 s and serially diluted as required. Gills were aseptically cut from three juvenile heads and pooled in a sterile container with pre-measured cooled diluent (ASWP). The gut of three juveniles was aseptically removed, gently scraped to empty any content, rinsed twice in 10 mL cooled diluent and transferred to a sterile container with pre-measured cooled diluent. Gills and gut samples were stored on ice until analysed. They were then weighed, diluted 10-fold, stomached (60 s), and serially diluted.

3.2.2 Microbiological analysis

Media used were spread plated and incubated aerobically. TVC were carried out on MA and presumptive *Vibrio* counts on TCBS after incubation at 15 °C (5-7 days). In analysis of probiotic feeds, strain 04-279 grew to larger colonies (yellowish) than 04-394 (white) on MA and could be specifically enumerated. Presumptive pseudomonad counts were evaluated in 2004 using the modified CFC-Pseudomonas medium (Oxoid, UK) according to Stanbridge and Board (1994) and incubated at 22 °C (3-4 days). LAB counts were enumerated on NAP agar incubated at 22 °C (3-4 days) and colonies confirmed by catalase test. Yeast counts in rotifers sampled in 2005 were obtained using DRBC agar following incubation at 22 °C (3 days).

A siderophore-indicative medium (chrome azurol S, CAS, Fluka; 15 °C, 5-7 days) was used to enumerate and characterise siderophore-producing bacteria in *Papers I* and *III*. The medium contained 1% casamino acids (Difco), 0.5% yeast extract, 3% agar (Difco), 0.5% NaCl supplemented with 30.24 g Pipes (Fluka), 6 g NaOH pellets, 60.5 mg chrome azurol S (Fluka), 0.01 mM Iron (III) chloride hexahydrate (Merck) and 72.9 mg hexamethyldisilazane (HDTMA, Sigma-Aldrich) based on Schwyn and Neilands (1987). In *Paper IV*, replica plating of grown TCBS plates was performed on blood agar containing 2% NaCl [BA-NaCl: blood agar base, Oxoid, supplemented with 5% (v/v) sheep blood and 1.5% (w/v) NaCl] at 15 °C for 5 days to estimate the number of haemolytic vibrios, i.e. colonies with a clear zone. In the juvenile feeding trial in 2006, total culturable haemolytic counts of seawater (days 0, 28 and 55) and juvenile gills (day 55) were evaluated on BA-NaCl after incubation at 15 °C for 5-7 days, counting the colonies with a clear zone. The detection limit was 10 CFU mL⁻¹ for rearing water and live feed, 20 CFU g⁻¹ for ova, older larvae, algal concentrate, dry feed, gills and gut, but 100 CFU g⁻¹ for younger larvae.

3.2.3 Bacterial isolation: phenotypic tests and grouping

Up to 571 bacterial isolates were maintained on MA while being investigated, as well as frozen at -25 °C in marine broth 2216 (MB, Difco) containing 30% (v/v) glycerol. Ten to 20 colonies were randomly taken from MA plates and 5-10 colonies from TCBS, CFC and NAP media. Grouping of the strains was carried out based on the following phenotypic traits as described in *Paper I*: colony appearance, Gram staining and potassium hydroxide reaction, cell shape, motility, catalase and oxidase tests, metabolism of glucose in a marine oxidation/fermentation medium and sensitivity to vibriostatic agent O/129 (150 µg).

Additional tests were performed for the strains isolated in 2005: growth of glucose-fermenting strains on TCBS (15 °C, 5 days), haemolytic activity on blood agar (BA-NaCl) at 15 °C (3-5 days) and ability to produce siderophores on CAS medium (15 °C, 3-5 days). Detection of a yellow halo around the single streak revealed the presence of siderophores, and its magnitude related to the importance of their production (-, +, ++, +++: from none to very much).

3.2.4 Partial 16S rRNA gene sequencing and genotypic classification

Genotypic identification of representative strains for the phenotypic groups obtained was achieved by partial 16S rRNA gene sequencing as described in *Paper I*. In *Paper III*, the sequences obtained for 257 isolates (154 in 2004 and 103 in 2005) were assembled into operational taxonomic units (OTUs) based on similarity calculation using a 98% cut-off value. Resulting OTUs were manually edited, followed by a BLAST alignment search on the NCBI server and the phylum of each sequence determined.

3.2.5 Differentiation within OTUs by API tests

Strain differentiation within OTUs including to some extent phenotypically different isolates from 2005 was performed using biochemical test kits, API 20 E and API 20 NE (BioMérieux, France) for glucose-fermenting (n=30) and non-fermenting isolates (n=52), respectively. Isolates were cultivated on MA at 22 °C for 3 days, pure colonies suspended in ASW, inoculated in API strips and incubated at 22 °C for 24 h. Results were recorded in binary form and analysed using hierarchical clustering (unweighted pair-group method with Euclidean distance type) in the statistical software NCSS 2000 (USA). API subtypes were defined at 0% dissimilarity (100% similarity) within the eight OTUs evaluated.

3.2.6 Detection of acylated homoserine lactones (AHLs)

Seventy-six isolates from 2005 were evaluated for their ability to produce AHLs for QS, using two different AHL monitor strains, *Chromobacterium violaceum* (CV026) and *Agrobacterium tumefaciens* NT1 (pZLR4). Isolates were streaked in parallel with the monitor strains onto LB agar (1% peptone, 0.5% yeast extract, 0.5% NaCl and 1.2% agar; modified from Bertani (1951), containing X-Gal (50 µg mL⁻¹, Promega, WI, USA) for pZLR4) with or without an antibiotic (20 µg mL⁻¹ kanamycin for CV026 and gentamycin for pZLR4), and incubated for 24-48 h at 22 °C. Colour development in the monitor strains revealed AHL production by isolates tested. Positive controls included *Yersinia ruckeri* F171-05 (Institute for Experimental Pathology, Keldur, University of Iceland) and C6-HSL (N-hexanoyl-L-homoserine lactone, Sigma-Aldrich) for CV026, and *V. anguillarum* F195-99 (Institute for Experimental Pathology, Keldur) and 3-oxo-C6-HSL (3-oxo-N-hexanoyl-L-homoserine lactone, Sigma-Aldrich) for pZLR4. Negative controls were the monitor strains themselves.

3.2.7 Chemical analysis of feed

During the probiotic feed storage trial and the juvenile trials, the moisture content [ISO 6496 (1983)] was evaluated in probiotic and control feeds by pooling replicate samples.

3.2.8 Sensory analysis of feed

At the end of the probiotic feed storage trial, sensory evaluation was conducted by four trained panellists assessing appearance and odour of feed types, control and probiotic, at all three storage temperatures. Following microbiological evaluation, the rest of the feed (n=3) was combined in coded glass petri dishes for each group and stored at room temperature to reach environmental temperature. Deviations from the control feed were recorded using the following scale: (5) no deviation, (4) on borderline, (3) little, (2) obvious and (1) much deviation.

3.3 Characterisation of probionts

3.3.1 Inhibitory activity towards fish pathogenic bacteria

Selected isolates (n=192) were tested for their inhibitory activity towards three fish pathogens using the well diffusion assay described by Vine *et al.* (2004). The pathogenic bacteria

included *V. anguillarum* F-139-03, *Aeromonas salmonicida* ssp. *achromogenes* F19-99 and *Aliivibrio* (*Vibrio*) *salmonicida* A8V-1-22, originally isolated from the head kidney of diseased fish on sheep blood agar (BA-NaCl). *V. anguillarum* and *Aeromonas salmonicida* were isolated from cod in Iceland and *Aliivibrio salmonicida* from salmon in Norway. Authenticity and purity of the pathogens were verified as described in **Paper I**. The strains were obtained from the biobank at the Institute for Experimental Pathology, Keldur, University of Iceland.

As described in **Paper I**, 188 representative bacterial phenotypes from the cod rearing environment and four bacterial isolates, retrieved earlier from either cod or salmon, were tested. Briefly, MA plates sawn with each pathogenic strain were perforated and MB-cultures of isolates (15 °C, 2 days) added to the wells. Following incubation (15 °C, 3 days), the inhibitory activity of an isolate towards a pathogen was evaluated by measuring the diameter (mm) of the clear zone around the well.

3.3.2 Production of extracellular inhibitory compounds

Production of extracellular inhibitory compounds was similarly assessed after filtration (0.22 µm) of inhibitory MB-cultures. Also, pH of the bacterial cultures was measured to assess the production of acidic compounds. Ability of inhibitory isolates to produce siderophores was evaluated on CAS medium (15 °C, 3-5 days). Detection of a yellow halo around a single streak revealed the presence of siderophores.

3.3.3 Growth characteristics of inhibitory and fish pathogenic bacteria

Optical density (OD) measurements in microtiter plates (96 wells) were used to study the growth behaviour of inhibitory and fish pathogenic bacteria in MB at 8-15-22-30-35 °C. Following pre-culturing at 15 °C in MB for 35 h, isolates were diluted and inoculated in MB in duplicate, reaching an initial inoculation level of about 10^{3-4} cells mL⁻¹. Absorbance (growth) was measured with a Titertek Multiskan Plus spectrophotometer (model MKII, Flow Laboratories) at 590 nm at regular intervals (0-2-5-13-22-28-37-48-53-61-72 h). Absorbance measurements at time 0 was used as the blank measurement for each respective well. The approximative optimal temperature was determined for each bacterial isolate based on the highest average absorbance value measured with respect to time. Time to reach an increase in

OD of 0.4 unit and length of the lag phase (time to detection, i.e. increasing OD) were approximated (in hours) from the curves for growth comparison among bacteria.

The ability of inhibitory bacteria to inhibit each other (interactive antagonistic activity) was tested by the well diffusion assay to verify whether promising bacteria could be used as a mixture. Further, haemolytic activity of selected inhibitory bacteria was assessed on sheep blood agar (BA-NaCl). Isolates were streaked, incubated at 16 °C for 3-5 days and examined for haemolytic activity.

3.3.4 Selection of isolates for adhesion assays

Selection of 6 inhibitory isolates for adhesion assays was done based on previous tests, mainly inhibitory spectrum and intensity, as well as preliminary identification and growth characteristics.

3.3.5 Adhesion assays

Adhesion capacity of bacterial isolates was evaluated using four fish cell lines: Epithelioma papulosum Cyprini (EPC) cells from *Cyprinus carpio*, Chinook Salmon Embryo (CHSE-214) cells from *Oncorhynchus tshawytscha*, Bluegill Fin (BF-2) cells from *Lepomis macrochirus* and Rainbow Trout Gonad (RTG-2) cells from *Oncorhynchus mykiss*, all a generous gift from the Danish Institute for Food and Veterinary Research in Aarhus. The bacteria tested were six inhibitory, non-haemolytic isolates that originated from the rearing water (04-279), algal concentrate (04-337 and 04-394), larval GIT (04-388), dry feed (04-683) and salmon intestine [CdV41, *Carnobacterium divergens* V41 (Pilet *et al.*, 1995)]. Other bacteria tested included two fish pathogens (*V. anguillarum* and *Aeromonas salmonicida*) and the human probiotic strain *Lactobacillus rhamnosus* GG (LGG, Valio Ltd, Finland) as a positive control.

Cell lines were cultured in Minimum Essential Medium (Earle's Salt, L-glutamine, NaHCO₃, Life Technologies, Gibco, USA) supplemented with 10% (v/v) foetal bovine serum (FBS, Life Technologies, Gibco) and 1% (v/v) streptomycin/penicillin (Life Technologies, Gibco). For maintenance, the cells were transferred (subcultivated) to new growth flasks (75 cm², Nunc, Denmark) every 28th day by trypsin/EDTA (Life Technologies, Gibco) treatment and split 1:3. After subcultivation, cells were incubated overnight at 28 °C to differentiate and form a monolayer followed by a 27-day incubation period at 16 °C.

For adhesion assays, cell lines were seeded at a concentration of 6×10^5 cells mL⁻¹ and dispensed into each 200 mm² well of a 24-well tissue culture plate (Nunc), incubated overnight at 28 °C followed by two days at 16 °C. An hour before the adhesion assay, the cell medium was replaced with fresh medium without antibiotics and cells were incubated at 16 °C. Overnight cultures of the bacterial strains, grown at 16 °C in MB, were centrifuged (10000 rpm for 2 min) and resuspended in the cell line growth medium without antibiotics to a final concentration of approximately 10^6 CFU mL⁻¹ and 1 mL of this suspension was added to each well of the tissue culture plate. After 1 h of incubation at 16 °C, the monolayers were washed three times with phosphate-buffered saline (PBS, pH 7.4) in order to remove non-adherent bacteria. The cells were lysed by addition of 0.2% (v/v) Triton-X100 (Merck) and the number of viable adherent bacteria were determined by plating serial dilutions onto MA. Colony-forming units were enumerated after aerobic incubation for up to one week at 16 °C and the adhesion capacity described as the percentage of bacteria adhered to the cell lines in relation to the total number of bacteria added. Each adhesion assay was conducted two times (two different passages) with duplicate determinations. Results are reported as average and standard deviation (SD) of four determinations.

3.3.6 Infectivity of selected probionts

A standard method was used to evaluate the infectivity of the two promising probionts (04-279 and 04-394). Cod juveniles (13 g) were inoculated intraperitoneally (i.p.) in duplicate with 0.1 mL of probiotic suspensions, serially diluted (up to 10^{-4}) in PBS. Control fish (n=18) received PBS only. Juveniles were evenly distributed in two tanks (150 L, 9 °C) with aerated seawater and hand fed once a day. They were observed for 7 days and then sacrificed with an overdose of TMS for external and internal pathological inspection. Head kidney samples from every fish were cultured on BA-NaCl (15 °C, 7 days). Colonies that grew on BA-NaCl and resembled those of the probionts used were purified (n=5) and analysed by partial 16S rRNA gene sequencing as described in *Paper I* for confirmation.

3.3.7 Enzymatic activity of selected probionts

The enzymatic activity of *Arthrobacter* and *Enterococcus* probionts was assessed using API ZYM (BioMérieux) with 19 enzymatic tests. Strains were cultivated on tryptic soy agar (Difco) at 22 °C for 2 days and pure colonies suspended in ASW. Optical density of bacterial

suspensions was measured, aiming at an absorbance value of 1 (Bioscreen C analyzer, wide band at 420-580 nm; Oy Growth Curves AB Ltd, Finland). The strip was inoculated according to manufacturer's instructions and incubated at 22 °C for 24 h, followed by the addition of supplements (ZYM A + B, BioMérieux) for colour development. Colour intensity (0-5) was recorded based on the manufacturer's interpretation scheme. Tests given 0-1 were regarded as negative, 2-3 considered low to moderate activity (10-20 nmol of hydrolysed substrate) and 4-5 indicated a strong reaction (30-40⁺ nmol).

3.4 Effects of probiotic treatments on cod rearing

3.4.1 Growth, survival and morphology of larvae

At the end of the larval trials in 2005 and 2006, all surviving larvae were concentrated into containers and counted for survival assessment in each silo, using the following formula (*Papers II-III-IV*):

$$\text{Survival (\%)} = (N_{\text{surviving+sampled larvae}} / N_{\text{initial larvae}}) \times 100.$$

Fifty (*Paper II*) or thirty (*Paper IV*) larvae were randomly taken from each silo and their length was measured using a stereomicroscope (WILD M3Z, Wild Heerbrugg Ltd, Switzerland). The presence of swim bladder was ascertained and any malformation noted (*Paper II*). Larvae from each silo were then divided into three or five subgroups of 10 larvae and dried for 48 h (80 °C, hotbox oven, Gallenkamp, UK). Mean dry weight and standard deviation were calculated from the measurements obtained.

3.4.2 Hypersalinity tolerance of larvae

Hypersalinity tolerance of differently treated larvae was evaluated 20 and 27 dph using the method described by Dhert *et al.* (1992). The test was performed at 7-8 °C in triplicate in 100-mL glass beakers, 7-10 larvae per beaker. The different salt concentrations were prepared by making solutions of sea salt (Aquarium Systems Inc, USA) and mixing with equal volumes of seawater to reach the final salt concentrations of 70 and 105 ppm. Normal seawater (32 ppm) was used for comparison. Larvae were carefully transferred from silos to beakers, pooling larvae from duplicate silos and trying to avoid stress by exposure to air. Dead larvae were counted and removed with 20-min intervals. On day 27, larvae from silos 1 and 3 were dominating in number because of lower survival in silos 2 and 4.

3.4.3 Larval lysate analysis

Larvae from each group were collected on 4, 14, 21 and 28 dph, quickly frozen and kept at -80 °C. The number of larvae collected depended on size, about 100 larvae in each pool or less as growth progressed. The samples were prepared for western blots (WB) and zymograms as described by Magnadottir *et al.* (2004). The protein content was estimated using a kit from Pierce Biotechnology Inc. (USA) based on the Bradford (1976) assay and adjusted to 2 mg mL⁻¹. The samples were divided in two parts. A protease inhibition cocktail (50-100 µL mL⁻¹, Sigma-Aldrich) was added to the WB sample and the other portion tested for enzyme activity using zymogram analysis.

For SDS-PAGE separation, lysates were diluted 1:1 in sample buffer containing SDS and mercaptoethanol, heated at 100 °C for 2 min. Western blotting was carried out according to Magnadottir *et al.* (2004) except for the last stages, i.e. secondary antibody and development. Five polyclonal mouse antibodies were used as primary antibodies: three were monospecific (anti-ApoLP A-I, anti-haemoglobin and anti-CRP) (Magnadottir *et al.*, 2004), one was raised against C3 and ApoLP A-I (Lange *et al.*, 2004a), and another against cod serum proteins (Magnadottir, 1998). Secondary antibody against mouse antibodies was conjugated with alkaline phosphatase (Dako A/S, Denmark). The blot was developed in a substrate buffer of 100 µg mL⁻¹ nitroblue tetrazolium, 60 µg mL⁻¹ 5-bromo-4-chloro-3-indonylphosphate and 4 mM MgCl₂ in 0.1 M ethanolamine-HCl buffer (pH 9.6).

Gelatinase and caseinase activity in larval lysates were determined by zymograms using SDS-PAGE gels (14% acrylamide with 0.1% gelatin or casein), loaded with 15 µL per channel (Magnadottir *et al.*, 2004). Samples were analysed unreduced and the SDS eluted from the gel after separation in 2% Triton-X-100 Tris buffer followed by 2 mM CaCl₂ Tris buffer before the gels were stained using Coomassie blue.

3.4.4 Juvenile survival, specific growth rate and feed conversion ratio

At the start of the trials in 2006, all juveniles were weighed in bulk, gathering the content of each tank in a pre-wet and weighed tray. On days 28 and 55, juveniles left in each tank were similarly weighed in bulk. This allowed for the weight gain assessment, correcting for dead and sampled fish. Juvenile specific growth rate (SGR), survival rate (%) and feed conversion ratio (FCR) were determined after 28 and 55 days as follows:

$$\text{SGR (\% day}^{-1}\text{)} = 100 \times (\ln(\text{average weight}_{\text{final}}) - \ln(\text{average weight}_{\text{initial}})) / \text{days in trial}$$

$$\text{Survival (\%)} = (\text{N}_{\text{surviving juveniles}} / \text{N}_{\text{initial juveniles}}) \times 100$$

FCR = total feed quantity given (g)/total weight gain (g)

3.5 Statistical analysis

Statistical analysis of larval and juvenile data was carried out using different tests in the NCSS 2000 software. The threshold level for significance was 0.05. Significance of treatments on adhesion data (*Paper I*), larval length and microbiological counts of rearing water in 2005 (*Paper II*) were assessed using the non-parametric test Kruskal-Wallis one-way ANOVA on ranks and the Kruskal-Wallis multiple comparison z-value test. Analysis of variance (one-way ANOVA) was applied to larval dry weight data in 2005 (*Paper II*) as well as larval and juvenile data from 2006 and rotifer culture data from 2005 and 2006, followed by a comparison of data with respect to treatments using the Tukey-Kramer multiple comparison test. Differences in larval and juvenile survival due to treatments were evaluated with the Fisher's exact test. Multivariate comparison of data in 2005 and 2006 (*Papers II-III-IV-V*) was conducted in the statistical program Unscrambler (Version 9.7, CAMO Software AS; Norway) with principal component analysis (PCA). Full cross validation was used and the data standardised with 1/SD (not standardised in *Paper III*). Cross-correlation among variables was also evaluated.

4 RESULTS

This chapter summarises the main findings of the research work undertaken to promote survival and larval development at early stages of cod rearing. Potential probionts were selected in 2004 from the natural microbiota of cod larviculture at an experimental station in Iceland, based on several *in vitro* tests (**Paper I**). Successively, three bacterial strains were tested as a mixture added to the rearing water from an early postfertilisation stage in the experimental cod hatchery in 2005. Two of the added bacteria were re-isolated in larvae at the end of the experiment 7 days post-treatment, contributing to increased survival, growth, vitality and microbiota control (**Paper II**).

In another study (**Paper III**), the dominant culturable microbiota was characterised as influenced by specific treatments during two spawning seasons (2004-2005). Current hatchery practices (ova disinfection and antibiotic treatment of unhealthy larvae) were evaluated (2004-2005), as well as specific putative probionts identified earlier (**Paper I**) and applied to ova and larvae (**Paper II**) or rotifers in 2005. Further, virulence-related phenotypic traits, including haemolysin, siderophores and AHLs, were studied among bacterial isolates from the trial conducted in 2005 and related to larval survival. In the spring of 2006 (**Paper IV**), the combined application of the two prospective probionts (*Arthrobacter bergerei* isolated from an algal concentrate and *Enterococcus thailandicus* from cod larval rearing water) was validated by their addition via the rearing water from the prehatch stage till 28-dph larvae. This bacterial bathing treatment was compared to groups fed rotifers supplemented with a commercial probiotic (Remus[®]) and those untreated. Effects of treatments on the microbial load in rearing systems, larval growth, survival and development were evaluated.

Finally, an intervention study was conducted at an early cod juvenile stage in the fall of 2006 to confirm the properties of the two probionts applied as a mixture or singly via dry feed to older fish for a shorter (28 days) or longer (55 days) period (**Paper V**). This involved the development of probiotic feed, its viability and quality assessment under different storage conditions (-20, 4 and 15 °C), as well as the evaluation of effects on juvenile growth, survival, feed conversion ratio, microbial load of rearing water, juvenile gills and gut.

4.1 Characterisation of probionts

A pool of 447 isolates, obtained from 34 samples of cod rearing water, ova, larvae, algal concentrate, rotifer and *Artemia* cultures, dry feed and juvenile gills, was used for probiont

characterisation. Phenotypic analysis discerned 185 groups, among which 158 isolates were selected for partial 16S rRNA gene sequencing. This analysis resulted in an identification of 60 OTUs, divided into 33 singletons and 27 contigs which contained 125 isolates. Representative isolates selected for the inhibition studies (n=188) included at least one isolate per OTU defined as well as 17 representatives of the phenotypic groups not analysed molecularly. Criteria for probiont selection were based on inhibitory activity towards three fish pathogens, growth characteristics as influenced by temperature, probiont interactive antagonistic activity, haemolytic activity, metabolite production, preliminary molecular identification and adhesion capacity using four fish cell lines. Additional isolates were studied: *Carnobacterium divergens* V41 (CdV41), a bacteriocin producer isolated from salmon intestine in France (Metivier *et al.*, 1998; Pilet *et al.*, 1995), and two isolates [*Carnobacterium piscicola* and *V. logei*-like (K1), commonly found in the experimental cod hatchery] from the biobank at the Institute for Experimental Pathology, Keldur. Further characterisation of the prospective probionts led to the assessment of their non-pathogenicity in cod juveniles by i.p. injection as well as their enzymatic potential using an *in vitro* test.

4.1.1 Occurrence and characteristics of inhibitory bacteria in cod rearing environment

Twenty-six out of 188 isolates showed inhibitory activity towards 1 to 3 pathogens (see Table 1 in **Paper I**), representing 13.8% of the selected isolates, with the dominance of Gram-positive bacteria (n=21 or 80.8%). Gram-positive isolates originated from larvae (10), algal concentrate (8), rearing water (1) and dry feed (2), and most of them inhibited *Aeromonas salmonicida* and *Aliivibrio salmonicida* (n=17 or 81%) while fewer showed inhibition towards *V. anguillarum* (n=9 or 42.9%). Gram-negative isolates from larvae (2), rearing water (2) and dry feed (1) only showed some inhibitory effect towards *Aliivibrio salmonicida* and *V. anguillarum*. Overall, the inhibition spectrum was broader and greater for Gram-positive than Gram-negative bacteria.

The inhibitory isolates were distributed within three bacterial phyla, listed by their importance (% of isolates): Firmicutes (69.2), γ -Proteobacteria (15.4) and Actinobacteria (11.5). One strain was not identified. Firmicutes included 6 OTUs, with the main inhibitory activity towards *Aliivibrio salmonicida* and *Aeromonas salmonicida*. Few Firmicutes inhibited *V. anguillarum* as well, but to a lesser extent, and these were LAB. The strain CdV41 was inhibitory towards the three pathogens tested. Isolates of γ -Proteobacteria belonged to 2 OTUs which showed inhibitory effect either towards *V. anguillarum* or

Aliivibrio salmonicida or both. Actinobacteria isolates (A-C2) strongly inhibited *V. anguillarum* though a clear zone was not totally obtained.

Acid production (pH drop ~ 1-1.5 unit) was evidenced in two Firmicutes OTUs and two isolates from two other OTUs. Otherwise, little or no pH decrease was observed among the isolates. Only one strain (04-342) was found to maintain its inhibitory activity after sterile-filtration of culture supernatant, indicating the presence of extracellular inhibitory compound(s). Siderophore production was detected among inhibitory Gram-negative isolates. Overall, it can be said that *Aliivibrio salmonicida* was the most sensitive pathogenic strain being inhibited by most isolates, while *V. anguillarum* was the least sensitive one. Some inhibitory Firmicutes isolates had the broadest antagonistic spectrum. Moreover, A-C2 had the greatest inhibition towards *V. anguillarum* and strain 04-342 towards *Aliivibrio salmonicida*. Several strains did inhibit *Aeromonas salmonicida* to a similar extent.

Table 6. Selection of bacterial isolates based on inhibition potential and growth characteristics

Isolate code	OTU code	Closest relative (Genbank, % similarity)	Inhibition intensity ^a (inhibited pathogens)	Time (h) to reach 0.4 OD increase at 15 °C ^b	Lag time ^c (h) at 8 °C	Haemolytic activity	Metabolites detected at 15 °C
04-279	A-C2	<i>Arthrobacter bergerei</i> (100)	+++ (1)	48	24	-	-
04-337	F-C2	<i>Alkalibacterium</i> sp. (99)	+ (2)	48	30	-	acid
04-342	F-C3	<i>Alkalibacterium olivapovliticus</i> (99)	++ (2)	66	No growth	-	cell-free activity
04-388	F-C1	<i>Enterococcus thailandicus</i> (100)	+ (2)	64	No growth	-	acid
04-394	F-C1	<i>Enterococcus thailandicus</i> (100)	++ (3)	42	60	-	-
04-538	P-C8	<i>V. splendidus</i> (100)	+ (1)	12	12	+	siderophore
04-673	F-C6	<i>Vagococcus fluvialis</i> (100)	+ (3)	56	36	(+)	acid
04-683	F-E	<i>Ent. faecium</i> (98)	+ (2)	40	36	-	acid
04-716	P-C8	<i>V. splendidus</i> (100)	+ (1)	12	12	+	siderophore
CdV41 ^d	-	<i>Carnobacterium divergens</i> (100)	+ (3)	24	24	-	(bacteriocin)

^a Inhibition intensity towards pathogens based on clear zone diameter (+ 10-19 mm, ++ 20-29 mm, +++ 30-39 mm); ^b Comparison to pathogens – time (hours) to reach 0.4 OD increase at 15 °C: *V. anguillarum* (18), *Aliivibrio salmonicida* (42), *Aeromonas salmonicida* (>72); ^c Approximative lag time (hours) derived from the growth curve at 8 °C (lowest in-house rearing temperature). For pathogens: *V. anguillarum* (30), *Aliivibrio salmonicida* (30), *Aeromonas salmonicida* (>72); ^d CdV41 is known to produce divercin V41, but this

characteristic was not verified in our study; OTU, operational taxonomic unit; OD, optical density. Genbank accession number: AJ609631 (A-C2), EF197994 (F-C1), AB167074 (F-C2), AF143511 (F-C3), AB272361 (F-C6), AF039901 (F-E); AJ874364 (P-C8), AY543036 (*Carnobacterium divergens* V41).

Proliferation of inhibitory isolates and the three fish pathogens over the temperature range 8-35 °C was followed by OD. Similar optimal growth temperatures were generally observed within each OTU (data not shown). Isolates with lower temperature requirements were found among A-C2 (*Arthrobacter* sp.) and P-C8 (*V. splendidus*) OTUs, while Firmicutes isolates grew fastest around 30 °C. The pathogenic bacteria *Aeromonas salmonicida* and *Aliivibrio salmonicida* showed restricted growth but proliferated at 15-30 °C and 8-15 °C, respectively. On the other hand, *V. anguillarum* grew over the whole temperature range, most rapidly at 22-30 °C.

Based on the inhibition potential of the isolates, their preliminary identification and growth characteristics, 10 isolates were selected for further analysis (Table 6). Their ability to inhibit each other as well as their haemolytic properties were evaluated. Isolates 04-538 and 04-716 (P-C8) were found to be β -hemolytic while isolate 04-673 (F-C6) was α -hemolytic. Table 6 also distinguishes between the fast- and slow-growing isolates, comparing them with pathogens at 15 °C, which is close to the upper rearing temperature for cod at early stage. The fastest growing pathogen was *V. anguillarum* (18 h), followed by *Aliivibrio salmonicida* (42 h) but *Aeromonas salmonicida* did not reach this level (>72 h). P-C8 isolates grew faster than all pathogens, while isolates CdV41, 04-683, 04-394, 04-279 and 04-337 grew faster than *Aliivibrio salmonicida* or at a similar rate. Testing at 8 °C, the lower in-house rearing temperature for cod at early stages, revealed that only few isolates (04-279, 04-538, 04-716 and CdV41) proliferated after a lag phase shorter than that observed for *V. anguillarum* and *Aliivibrio salmonicida* (30-32 h). This overview of the main characteristics of the pre-selected isolates indicates the rarity of bacteria with maximal properties. Little antagonism was observed among pre-selected isolates (data not shown), indicating the possibility to use most of them as mixtures in probiotic *in vivo* trials.

4.1.2 Adhesion assays

All pre-selected and non-haemolytic isolates were tested for adhesion capacity to four fish cell lines (**Paper I**) and found to adhere best to the CHSE cells (Table 7). Strain 04-342 grew poorly and could not be tested. The pathogenic strain *Aeromonas salmonicida* (Keldur265-87) adhered well to all cell lines tested. Adhesion capacity of isolate 04-279 differed among the

cell lines, with no adherence to the BF cells. Isolates 04-388 and CdV41 adhered to all cells, except the EPC cells. Most variation in adhesion among isolates was to the EPC cells. Overall, the results indicate that three of the LAB isolates (04-683, 04-394 and CdV41) showed good adhesion capacity to at least three of the cell lines used.

Table 7. Adherence of selected bacterial isolates to four fish cell lines *in vitro* at 16 °C

Bacterial isolates	Closest relative	Cell lines			
		CHSE ^a	BF ^b	RTG ^c	EPC ^d
04-683	<i>Enterococcus faecium</i>	+++	+++	+++	+++
04-394	<i>Enterococcus thailandicus</i>	+++	+++	+++	++
CdV41	<i>Carnobacterium divergens</i>	+++	+++	+++	0
04-388	<i>Enterococcus thailandicus</i>	+++	++	++	0
04-337	<i>Alkalibacterium</i> sp.	++	++	+	++
04-279	<i>Arthrobacter bergerei</i>	+++	0	++	+
Keldur265-87	<i>Aeromonas salmonicida</i> ssp. <i>achromogenes</i>	+++	+++	++	++

Adherence expressed as +++ (> 5% adhering), ++ (1 to 5%), + (0.5 to 1%) and 0 (< 0.5%).

^a CHSE-214, Chinook Salmon Embryo cells from *Oncorhynchus tshawytscha*.

^b BF-2, Bluegill Fin cells from *Lepomis macrochirus*.

^c RTG-2, Rainbow Trout Gonad cells from *Oncorhynchus mykiss*.

^d EPC, Epithelioma papulosum Cyprini cells from *Cyprinus carpio*.

4.1.3 Selection of putative probionts

Five isolates were retained for future investigations. Isolate 04-394 from the algal concentrate has a broad and good inhibitory potential, with acceptable growth at 15 °C and good adhesion capacity. Isolate CdV41 from salmon intestine has a broad inhibition spectrum, very good growth at 15 °C and good adhesion capacity. Isolate 04-279 from larval rearing water has a restricted inhibitory spectrum, inhibiting only *V. anguillarum* but with great intensity, with acceptable growth at 15 °C and some adhesion capacity. A fourth isolate (04-683 from dry feed) has a similar temperature requirement as 04-394, a lesser inhibitory potential but good adhesion capacity. Despite the lack of data on adhesion capacity for strain 04-342 from the algal concentrate, it was also selected because of its great antagonistic activity towards *Aliivibrio salmonicida* but its higher temperature requirement should direct its use above 15 °C. Due to the low temperature tolerance of the four other isolates, they could be applied in mixture as a rearing water supplement to promote complementary action and control the microbiota of cod rearing systems. The addition of LAB or other probionts to control the

bacterial development in live feed is a promising area. Rotifers and *Artemia* are filter-feeders and therefore appropriate vectors for probiont delivery to fish larvae.

4.1.4 Infectivity of two prospective probionts

The infectivity of two putative probionts, *Arthrobacter bergerei* (04-279) and *Enterococcus thailandicus* (04-394) that led to promising results in the cod hatchery, was verified by i.p. inoculation of cod juveniles followed by their observation for one week and their subsequent sacrifice (**Paper IV**). Gross pathology revealed no signs of infection but the probionts were isolated from head kidney samples of fish inoculated. The lowest inoculum in fish resulting in isolation from head kidney was 3.9×10^3 CFU for strain 04-394 and 6.5×10^4 CFU for 04-279. Isolated colonies were confirmed as injected probionts by partial 16S rRNA gene sequencing.

4.1.5 Enzymatic activity of two prospective probionts

Testing for 19 different enzymes revealed the activity of 7 common enzymes in both prospective probionts, as shown in Table 8 listing their relative intensity and related target molecules (**Paper IV**).

Table 8. Enzymatic potential of the two prospective probionts, *Arthrobacter bergerei* (04-279) and *Enterococcus thailandicus* (04-394)

Enzymes	Probionts		Enzymatic reaction ^a
	04-279	04-394	
alkaline phosphatase	2	1	Removes phosphate groups from many types of molecules
C4-esterase	1	2	Hydrolyses esters to butyric acid
C8-esterase lipase	3	3	Hydrolyses esters to caprylic acid
leucine arylamidase	5	5	Liberates leucine, an essential amino acid
valine arylamidase	4	2	Liberates valine, an essential amino acid
cystine arylamidase	2	3	Liberates cystine, a source of cysteine
α -chymotrypsin	1	2	Cleaves peptides to liberate tyrosine, tryptophan, phenylalanine, leucine
acid phosphatase	2	5	Liberates attached phosphate groups from other molecules
β -galactosidase	0	5	Hydrolyses β -galactosides
α -glucosidase	5	3	Breaks down complex carbohydrates (starch, glycogen)
α -mannosidase	4	1	Break down complex sugars (α -mannose)
Total enzyme score	29	32	

Scores: 0-1 = negative; 2 = low; 3 = moderate; 4-5 = strong reaction. ^a Ref: Dixon and Webb (1979).

Overall, strain 04-394 scored slightly higher (32) than 04-279 (29). Both strains produced a range of digestive enzymes, showed moderate lipolytic activity and good proteolytic potential capable of releasing important essential amino acids. Further they could break down complex molecules, like polysaccharides, carbohydrates or sugars.

4.2 Effects of bacterial bathing of ova and larvae on microbial load, larval performance and development

The following *in vivo* studies were conducted in 2005 and 2006 (*Papers II and IV*, respectively) at an experimental cod hatchery, from the postfertilisation stage to the mid-larval stage. The studies report on the effects of putative probionts applied via the rearing water (10^{6-7} CFU mL⁻¹) as a mixture on the microbial load of rearing systems, viability of larvae evaluated by hypersalinity tolerance, as well as larval survival, growth and development. Larval development was assessed by proteolytic activity of larval lysates and immunological analysis of important proteins for cod at early stage: apolipoprotein A-I, haemoglobin, C-reactive protein, C3 and cod serum proteins. The probiont mixture contained three or two bacterial strains (04-279, 04-394, and CdV41 additionally in 2005).

4.2.1 Microbiological status in larviculture rearing units

Table 9 compares the data obtained for the control and bathed treatments from the postfertilisation stage to the mid-larval stage during both hatchery trials. Analysis of the untreated seawater flowing into the ova incubators revealed its low culturable microbial load (TVC, 2.5 log CFU mL⁻¹) with no detectable vibrios (<0.3 log CFU mL⁻¹). The mean culturable microbial load of the rearing water became similar at pre- and posthatch stages, independently of the treatment received, ranging between 3.4-3.9 log CFU mL⁻¹ and indicating a 10-fold increase in the seawater. Presumptive *Vibrio* load of rearing water was low or undetected prior to hatch but reached a mean value of 2 log CFU mL⁻¹ posthatch in most larval silos. Replica plating of TCBS medium onto blood agar (BA-NaCl) during the 2006 trial demonstrated the presence of haemolytic vibrios from 4 dph in all silos (data not shown). Variations were observed in counts between sampling points early and late in the periods studied, and most likely coincided with the onset of rotifer feeding (3 dph), followed by the increase in water flow increased and an organic build-up in the rearing units as time progressed (see Figure 4 for an example).

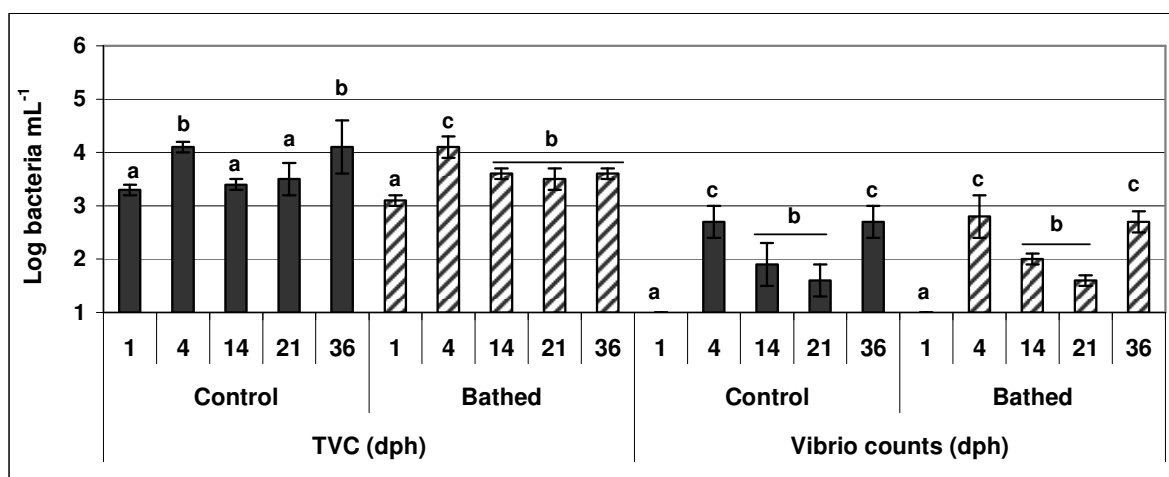


Figure 4. Microbial load (log CFU mL⁻¹) of rearing water in differently treated larval silos (n=4) in 2006

Control, untreated larvae; Bathed, larvae bathed regularly with two probionts [*Arthrobacter bergerei* (04-279) and *Enterococcus thailandicus* (04-394)]. Different letters indicate significant differences among sampling points per treatment ($P < 0.05$); dph, days posthatch. Count below 2.3 log CFU mL⁻¹ is an estimate.

In 2005, mean concentration of the bacterial mixture in the rearing water during ova bathing was 7.3 ± 0.2 log CFU mL⁻¹. Presence of added LAB in incubator rearing water was detected up to 3 days post-treatment, but not after a longer period (data not shown). Analysis of the algal concentrate added daily to the larval silos revealed low levels of moulds and yeasts (3.3 and 3.6 log CFU g⁻¹, respectively), but high LAB levels (6.7 log CFU g⁻¹) which could be detected in the rearing water (about 2 log CFU mL⁻¹) sampled after supplementation. Otherwise LAB counts in the rearing water were generally low or below detection level, even in the bathed larval silos (5 and 7 days post-treatment; data not shown).

Regular bathing of ova (1-4-7 dpf) in 2005 apparently led to a lower microbial build-up than on untreated ova, as evaluated 11 dph (Table 9). Bacterial counts of bathed ova on NAP medium showed that LAB represented almost 1% of the total culturable microbiota (TVC, data not shown), indicating the presumable establishment of added LAB on ova. Presumptive *Vibrio* counts of ova were low, representing about 0.01% of TVC, but vibrios isolated from control ova on TCBS agar were haemolytic. Higher *Vibrio* levels (almost 10% of TVC) were found on control ova (10 dpf) in 2006, most of them being haemolytic. In 2005, a similar microbial load was found on larvae 2 dph as for ova 11 dpf (data not shown). Higher *Vibrio* levels were found on control larvae than bathed, dominated by haemolytic vibrios. The presence of one (04-394) of the added bacteria was confirmed 2 dph on bathed larvae using partial 16S rRNA gene sequencing, i.e. a week after the latter ova

treatment at 7 dpf. First-feeding started 3 dph. The analysis of larval GIT 8 dph onwards showed that TVC were similar among the differently treated larval groups, as demonstrated by the mean counts over the period studied, while *Vibrio* and LAB counts varied (Table 9). It is noteworthy that *Vibrio* counts obtained on TCBS medium were occasionally lower than those calculated on the basis of identified colonies from TVC plates and their proportion to the total culturable microbiota. The added strain 04-279 was present at high levels (5.7-5.8 log CFU g⁻¹) in bathed larvae 27 dph (silos 1-2, i.e. 7 days post-treatment), as confirmed by partial sequencing. Further, a high LAB level was found in treated larvae from silo 1, similarly confirmed to be strain 04-394. The added strain CdV41 was not isolated from the GI microbiota of bathed larvae.

Table 9. Summary of data for hatchery trials conducted in 2005 and 2006 comparing bacterial bathing treatment of ova and larvae with untreated (control) groups

Treatment		2005				2006	
		Control		Bathed ¹		Control	Bathed ¹
Prehatch stage	Rearing water ² (log CFU mL ⁻¹)					(10 dpf) ⁶	
	TVC	3.7 (0.5)		3.4 (0.5)		3.8 (0.1)	
	<i>Vibrio</i> counts	TFTC		TFTC		TFTC	
	Ova (log CFU g ⁻¹)	(11 dpf)		(11 dpf)		(10 dpf) ⁶	
	TVC	5.9		5.2		5.3 (0.4)	
	<i>Vibrio</i> counts	1.9		NA		4.1 (0.5)	
Posthatch stage	Silo	S3	S4	S1	S2	S5-S8	S1-S4
	Rearing water ² (log CFU mL ⁻¹)						
	TVC	3.8 (0.3)	3.6	3.8 (0.4)	3.9 (0.1)	3.7 (0.4)	3.6 (0.4)
	<i>Vibrio</i> counts	2.0 (0.6)	1.7	2.1 (0.5)	2.0 (0.5)	2.2 (0.7)	2.2 (0.6)
	Larvae ³ (log CFU g ⁻¹)						
	TVC	6.6*	6.6	6.5	6.4	6.2 ^b (0.2)	8.1 ^a (0.4)
	LAB counts	2.0	ND	5.1 ⁷	ND	1.7 ^b (0.2)	4.1 ^a (1.1) ⁷
	<i>Vibrio</i> counts	5.4	3.9	4.7	4.9	5.8 (0.2)	6.0 (0.2)
	Calculated <i>Vibrio</i> ⁴	5.9	5.6	<5.5	5.4	NA	NA
	%TVC as 04-279	0.005%	ND	20%	20%	ND	0.01% ⁸
	%TVC as 04-394	0.0025%	ND	10%	ND	ND	0.01% ⁸
	%TVC as CdV41	ND	ND	ND	ND	NU	NU
	Survival rate ⁵ (%)	21.6 ^b	5.2 ^d	42.1 ^a	11.2 ^c	24.6 ^a (6.5)	20.4 ^b (6.2)
	Length (mm)	9.2 (0.4)	NA	9.3 (1.0)	9.1 (0.7)	10.7 ^b (0.5)	11.3 ^a (0.6)
	Dry weight (mg)	0.55 ^b (0.02)	NA	0.66 ^a (0.04)	0.56 ^b (0.03)	0.98 ^b (0.14)	1.21 ^a (0.12)

¹ Bacterial bathing treatment: a mixture of bacterial strains [*Arthrobacter bergerei* (04-279), *Enterococcus thailandicus* (04-394), and *Carnobacterium divergens* V41 (CdV41) only used in 2005]; ² Mean count of rearing water sampled over rearing period; ³ Larvae sampled 27 dph in 2005 and 36 dph in 2006; ⁴ *Vibrio* count calculated based on the phenotypic and genotypic profiles of isolated colonies (n=10 from MA); ⁵ Survival rate includes larvae sampled posthatch and those surviving on the last sampling day; ⁶ Mean count of rearing water or ova sampled 10 dpf from ova incubators before disinfection and bathing treatment; ⁷ Time elapsed at sampling from the last bacterial bathing treatment: 7 days in 2005 and 8 days in 2006; ⁸ Estimated based on the colonies distinguished on NAP medium; * 20% of TVC were haemolytic vibrios. TFTC, too few to count; NA, not available; ND, not detected; NU, not used; dpf, days postfertilisation. Standard deviation given in parenthesis for mean values. Different letters indicate significant ($P<0.05$) difference between treatments in 2005 or 2006.

In 2006, larval GI counts were significantly higher in bathed than control larvae. *Vibrio* counts of differently treated larvae did not vary significantly but they all increased significantly 36 dph, dominating the GI microbiota of control larvae (40% of TVC). Haemolytic vibrios were found in all larval samples but their proportion to the total culturable microbiota differed among treatments, $0.02 \pm 0.02\%$ and $1.06 \pm 0.21\%$ of TVC in bathed and control larvae, respectively. Added probionts in bathed larvae (36 dph, 8 days post-treatment) accounted for about 0.01% of the total culturable GI microbiota or $2.0 \pm 1.2 \log \text{CFU larva}^{-1}$ for strain 04-394 and $2.1 \pm 0.2 \log \text{CFU larva}^{-1}$ for strain 04-279.

4.2.2 Hypersalinity tolerance of larvae

Tolerance to extreme conditions was tested by exposing the study groups to hypersalinity (Dhert's test). On 20 dph, both groups tolerated 70 ppm salinity similarly after 60 min exposure ($P=0.184$), while at 105 ppm 100% mortality was seen (data not shown). A week later (Table 10) similar mortalities were observed in 105 ppm at all times, with 100% death in both groups after 60 min. After 90 min exposure to 70 ppm salinity, bathed larvae showed significantly better tolerance than the control group ($P=0.004$).

Table 10. Accumulated larval death (%) evaluated 27 dph by Dhert's test for hypersalinity tolerance of bathed and control larvae ^a

Treatment time (min)	Accumulated death (%) at salinity of								
	32 ppm			70 ppm			105 ppm		
	Control	Bathed	<i>P</i>	Control	Bathed	<i>P</i>	Control	Bathed	<i>P</i>
20	0	3.6	1.000	0	0		13.8	14.3	1.000
40	0	7.1	0.513	36	0	<0.001	93.1	82.1	0.253
60	0	7.1	0.513	40	30.8	0.161	100	100	
90	5.6	10.7	0.646	80	38.5	0.004	100	100	

^a Pooled control larvae originated mainly from S3 (94.2%); bathed larvae from S1 (78%).
P Probability level obtained by Fisher's exact test.

4.2.3 Larval survival, growth and development

On the last sampling day, the effect of the bacterial bathing treatment on larval performance was compared with control larvae. Table 9 provides the larval survival rate and growth data for larvae of different age, 27 dph (2005) and 36 dph (2006). In 2005, larval survival was found to differ significantly ($P<0.001$) among silos, i.e. intra- and inter-treatments. Overall comparison of survival between treatments reveals that it was significantly higher for bathed larvae than for control ones ($P<0.001$). On average, larvae were twice as likely to survive if they received the bacterial treatment regularly from the postfertilised stage (RR=1.98; 95% CI: 1.82 to 2.17). However, this effect was not observed in 2006. Some variation was seen among the four replicates of the control and bathed treatments (CV=0.26 and 0.30, respectively), but the statistical analysis indicated a significant difference ($P<0.001$) in survival rate between control and bathed silos. On average, untreated larvae were 1.2 time (relative ratio, RR) as likely to survive compared to bathed larvae.

Considering the treatment effect on growth (Table 9), a faster development was evidenced in bathed larvae in both trials compared with untreated ones, with significantly higher ($P<0.05$) average dry weight and length (only in 2006). Bathed larvae were on average 23.5% heavier and 5.6% longer than control larvae (36 dph) in 2006.

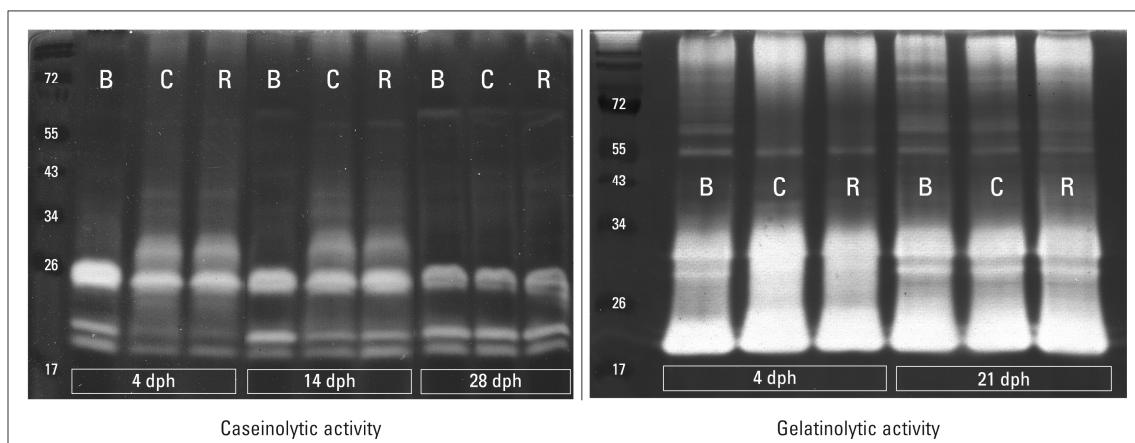


Figure 5. Zymograms on lysates from differently treated larvae pooled on 4, 14, 21 or 28 dph showing caseinolytic and gelatinolytic activity B, larvae bathed with both probionts [*Arthrobacter bergerei* (04-279) and *Enterococcus thailandicus* (04-394)]; C, control larvae; R, larvae fed rotifers supplemented with Remus®. First lane with prestained standards (kDa).

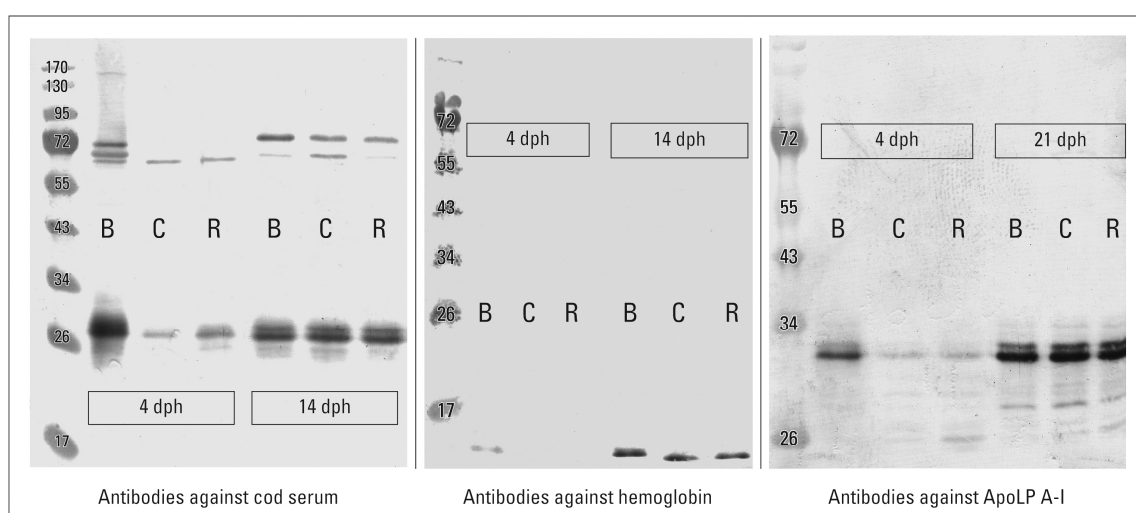


Figure 6. Western blots (WB) on lysates from differently treated larvae pooled on 4, 14 or 21 dph. WB probed with antibodies raised against cod serum proteins, haemoglobin and apolipoprotein (ApoLP) A-I B, larvae bathed with both probionts [*Arthrobacter bergerei* (04-279) and *Enterococcus thailandicus* (04-394)]; C, control larvae; R, larvae fed rotifers supplemented with Remus®. First lane with prestained standards (kDa).

Analysis of larval lysates for proteolytic activity and immunological proteins of importance in cod at early stage also indicated a faster development in bathed larvae in 2006. Zymograms for caseinolytic and gelatinolytic activity in larval lysates (Figure 5) showed increased proteolytic activity in the bathed group as compared to the control 4 dph, while little difference was seen at other sampling times. Similarly, Figure 6 reveals that the antibodies

raised against apolipoprotein (apoLP) A-I detected on 4 dph stronger bands in the bathed than the control group. Antibodies against cod serum proteins showed on 4 dph a broader band around 26-34 kDa in the bathed group as well as bands only detected in the bathed larvae sample (2 bands at 72 kDa and one at 130-170 kDa). Antibodies raised against haemoglobin detected a band in bathed larvae 4 dph, which corresponded to that found in the control 10 days later. Western blots probed with antibodies against CRP and C3 were negative (results not shown).

4.2.4 Overall assessment of larval parameters evaluated

Evaluation of the relationship between the parameters measured 27 dph revealed that survival rate was highly correlated to LAB and 04-394 counts ($r=0.988$ and 0.908 , respectively) as well as dry weight and length ($r=0.904$ and 0.983 , respectively), but to a lesser extent to 04-279 counts ($r=0.618$). Negative correlation was seen between calculated *Vibrio* counts and dry weight ($r=-0.948$), 04-394 counts ($r=-0.915$) and survival ($r=-0.711$). This is demonstrated in Figure 7, showing the bi-plot of PCA analysis between the several parameters.

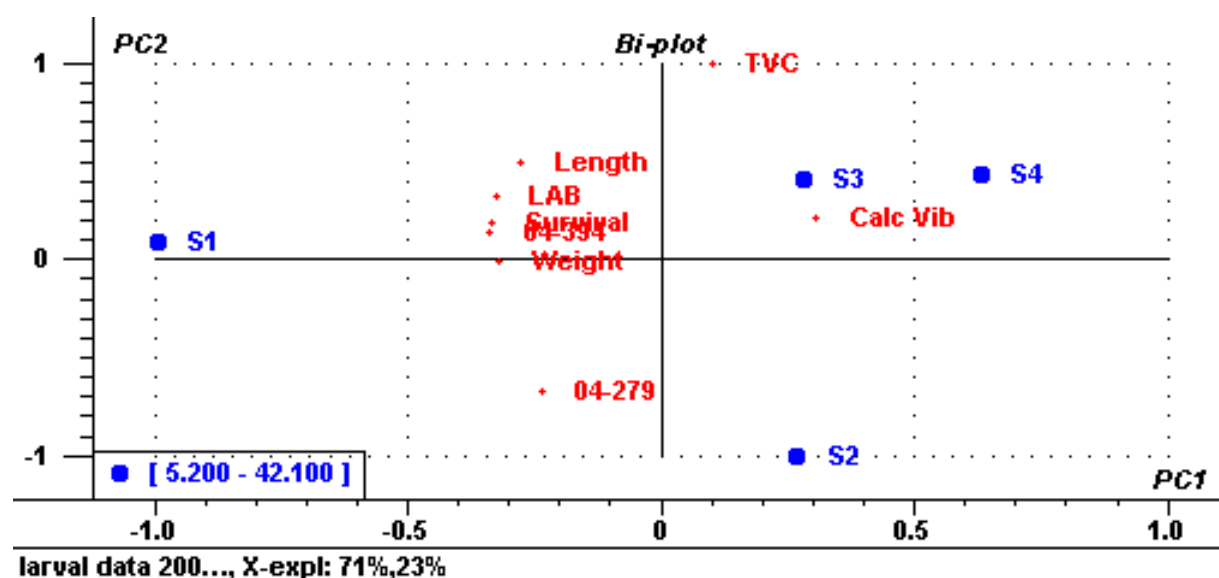


Figure 7. Effects of treatments on larval performance, development and microbial GI load: scores (larval samples with dot) and loadings (variables measured) of a principle component analysis (PCA)

Samples: S1 and S2 are bathed larvae, S3 and S4 are untreated larvae (control). Variables: larval survival rate, dry weight and length; TVC, Calc Vib (calculated *Vibrio* counts), LAB counts, estimated 04-279 and 04-394 counts.

The first principal component (PC1) explains 71% of the variation between the samples, with higher larval performance (survival rate and related parameters: 04-394 and LAB counts, dry weight and length) positioned to the left in opposition to higher calculated *Vibrio* counts to the right. The second component (PC2) explains 23% of the variation and differentiates between samples with similar TVC (upper part) and those containing strain 04-279 (lower part). This clarifies the position of S1-bathed larvae on the far left in contrast to S3- and S4-untreated larvae in the upper right quadrant and S2-bathed larvae in the lower one.

4.3 Effects of rotifer treatments on microbial load, larval survival and development

This part of the study investigated the microbial load of rotifer batch cultures as influenced by enrichment, rinsing or microbial supplementation, and how the latter affected larval survival and development (*Papers III and IV*). Further, disinfection of rotifer culture and enrichment treatments were evaluated in the attempt to reduce the culturable microbial load.

4.3.1 Performance of microbial treatments applied to rotifer culture

The effect of microbial treatments on the microbiological quality of rotifer culture was evaluated through several trials performed in duplicate over a 74-h period. Different microbial treatments were tested, involving two bacterial strains (04-342 isolated from an algal supplement and 04-683 from dry feed) at an initial concentration of 10^{4-5} CFU mL⁻¹ and two commercial probiotic products, Remus[®] (0.1 g L⁻¹) and Levucell SB20[®] (10^{5-6} CFU mL⁻¹ added at time 3, 24, 48, 69 h). A neomycin pre-treatment (50 ppm) was applied for 2 h on the rotifer culture which led to a significant tenfold decrease in TVC and presumptive *Vibrio* counts prior to the addition of putative probionts ($P < 0.05$). Figure 8 shows the microbial development during the whole experiment. No significant difference was observed among treatments for TVC or *Vibrio* counts (from time 0) at each sampling point, but as a general trend *Vibrio* counts increased with time and during enrichment except for treatments including strain 04-683 or 04-342 which were at or below 10^4 CFU mL⁻¹ at the end of the experiment. The effect of adding the probiotic yeast (Levucell SB20[®]) to rotifer cultures including these bacterial strains was unclear. Initial yeast level (5.4-5.7 log CFU mL⁻¹) had dropped 100-fold after 20-22 h post-supplementation, but because of its addition after 69 h it

was found at higher levels (4.8-4.9 log CFU mL⁻¹) at the end of the experiment. Levels of strain 04-683 could be followed using NAP medium (4.2-4.3 log CFU mL⁻¹ at time 0), being slowly reduced with culture time and found to be at or below 10³ CFU mL⁻¹ after 74 h (data not shown). No selective medium was available for 04-342 enumeration.

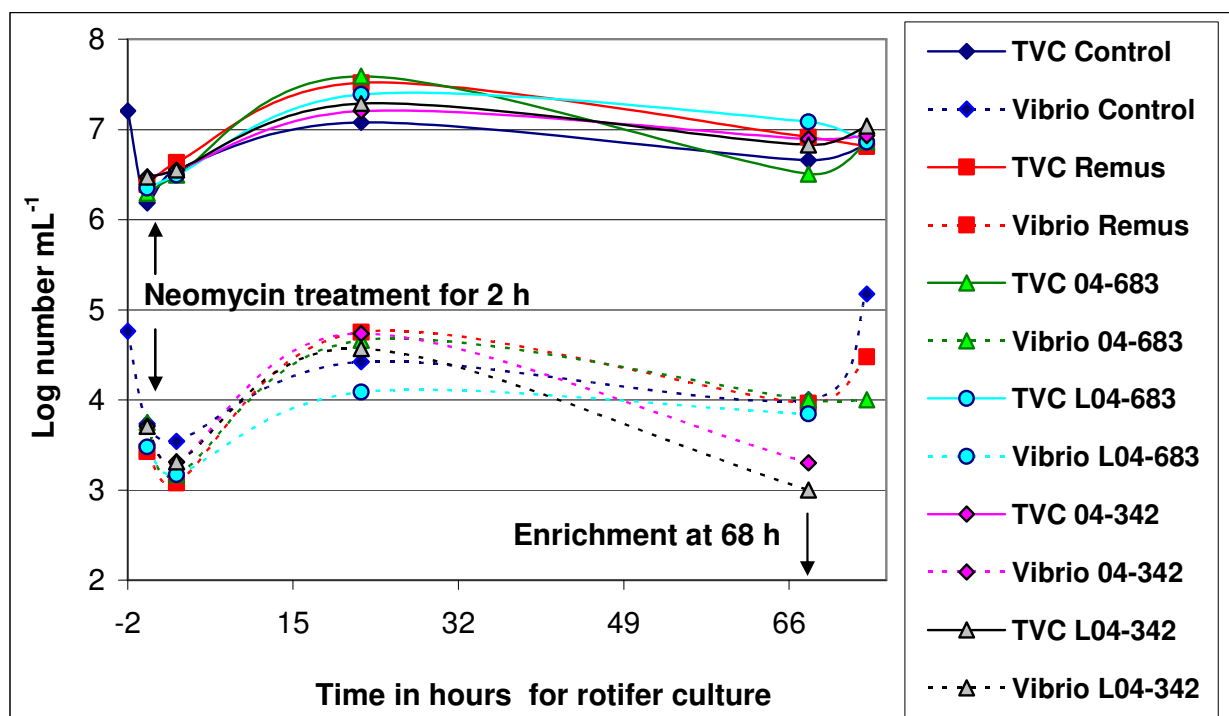


Figure 8. Effect of microbial treatments on microbiological quality of enriched rotifer cultures, simulating a batch culture. Putative probionts added to rotifer cultures (500 individuals/800 mL) following a 2-h neomycin treatment.

Average TVC (full line) and presumptive *Vibrio* counts (dashed line) are shown (n=2). Microbial treatments: 04-683 ± Levucell (L), 04-342 ± Levucell (L) or Remus. Presumptive *Vibrio* counts of L04-683, 04-342 and L04-342 were below 10⁴ CFU mL⁻¹ after a 6-h enrichment period (experiment end).

4.3.2 Microbiological status in rearing systems and impact of rotifer treatments

Rotifers cultured in batch generally carry a high microbial load. Microbiological analysis of enriched rotifers over the three cod rearing seasons studied revealed a total culturable microbial load of 7.2 ± 0.2 log CFU mL⁻¹ with a high proportion of vibrios (5.5 ± 0.2 log CFU mL⁻¹) but low levels of indigenous yeasts and LAB (about 0.01% of TVC). In 2005, rinsing of enriched rotifers was shown to decrease almost 10-fold TVC and yeast counts, while a lesser reduction (0.6 log) was observed for LAB and *Vibrio* counts (Figure 9).

In 2005, a study investigated whether feeding larvae once a day with treated rotifers could enhance their performance (*Paper III*). Treated rotifers were inoculated upon enrichment with a probiotic yeast (Levucell SB20[®]) and two putative bacterial probionts (04-342 and 04-683), resulting in an initial cell concentration of 10^{6-7} CFU mL⁻¹ rotifer culture. Microbial development in untreated and treated rotifers at different production steps is compared in Figure 9. Little change in total culturable microbial load occurred during enrichment of untreated rotifers while a loss of added LAB (10-fold) and yeasts (100-fold) was observed in treated rotifers after enrichment concurrently to a 10-fold *Vibrio* increase. Rinsing reduced the microbial load of both types of enriched rotifers.

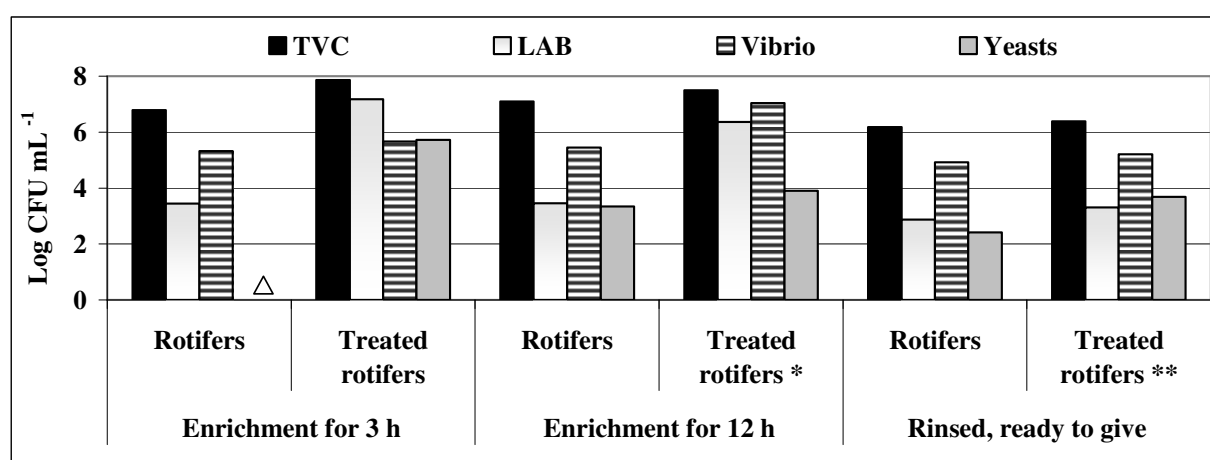


Figure 9. Microbial load (log CFU mL⁻¹) of rotifer culture as influenced by enrichment time, microbial supplements and rinsing prior to larval feeding

Treated rotifers received a microbial mixture during enrichment (12 h), including two bacterial strains [*Alkalibacterium olivapovliticus* (04-342) and *Enterococcus faecium* (04-683)], and a probiotic yeast (Levucell SB20). Δ indicates that yeast count is not available; * shows counts of treated rotifers given un-rinsed to S5 and S6 larvae; ** shows counts of treated rotifers given after rinsing to S5 and S6 larvae 20 dph and onwards.

A successive study was conducted in 2006 to evaluate the effect of Pyceze[®] treatments (0-25-50-100 ppm for 30 min) on the microbiological quality and viability of rotifer cultures. Figure 10 shows that Pyceze[®] had little effect on TVC but increasing concentrations affected *Vibrio* levels significantly ($P < 0.05$). The greatest reduction (~ 100-fold) in *Vibrio* counts was achieved with 50 and 100 ppm, but the higher concentration reduced rotifer viability by 50%. In a second trial, the use of Remus[®] during a 12-h enrichment of pre-treated rotifers (rinsed and disinfected with 0 and 75 ppm Pyceze[®]) was evaluated. Microbial counts were assessed after the enrichment. Use of Remus[®] during enrichment generally contributed to lower TVC

and *Vibrio* counts. The results may also indicate that Pyceze[®] disinfection of rotifers prior to enrichment may select for more resistant bacteria that will further grow during enrichment. Therefore, the use of Remus[®] with no pre-treatment gave optimal results. These findings led to the Remus-treatment of rotifers tested in a larval probiotic trial in 2006 (*Paper IV*). For practical reasons, the experimental station did not enrich the rotifers with Remus[®], but instead performed a rotifer boosting treatment (10 g ration⁻¹) of 30 min after enrichment, feeding larvae with un-rinsed Remus-rotifers. A similar treatment performed earlier was shown to be successful (Sveinsdottir *et al.*, 2009).

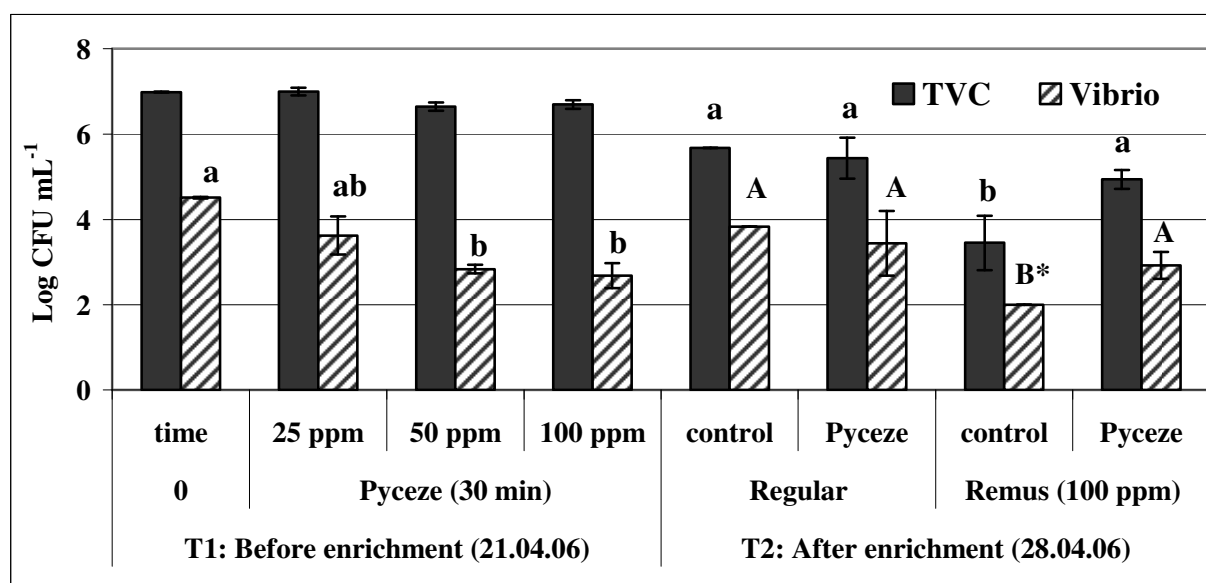


Figure 10. Effect of Pyceze[®] treatments prior to rotifer enrichment as well as Remus[®] supplementation during a 12-h enrichment on the microbiological quality of rotifers
 * indicates that *Vibrio* counts were < 2 log CFU mL⁻¹; T1, trial 1 conducted on batch-cultured rotifers; T2, trial 2 involved pre-treatments (rinsing $\pm$ 75 ppm Pyceze[®] for 30 min) of batch-cultured rotifers followed by enrichment.
 Different letters indicate significant ($P < 0.05$) difference between treatments within a trial.

Therefore, two larval trials tested putative probiotic rotifers. Mean microbial load of larval rearing water did not differ significantly between control (see Table 9) and treated groups (data not shown) over the periods studied. Larvae fed treated rotifers (S5+6) in 2005 fared poorly 20 dph, with high *Vibrio* counts. TVC of untreated larvae (6.6 log CFU g⁻¹) and those fed treated rotifers (6.4 log CFU g⁻¹) were comparable, while *Vibrio* counts were 10-fold higher in treated larvae compared to untreated ones. This is most likely related to the high *Vibrio* load of treated rotifers, given un-rinsed to the larvae (Figure 9). In 2006, analysis

of larval microbial load indicated no significant differences in TVC and *Vibrio* counts between untreated and Remus-treated larvae, despite the fact that the latter ones fared significantly less well. On the last sampling day (36 dph), vibrios were prevailing among the GI microbiota of untreated (40% of TVC) and Remus-treated (79% of TVC) larvae. Further, the proportion of haemolytic vibrios in larval samples with respect to their total culturable microbiota was significantly higher in Remus-treated larvae ($30.7 \pm 14.9\%$) than in control ($1.06 \pm 0.21\%$) larvae. Interestingly, counts of Remus-treated rotifers did not differ from untreated ones (log CFU mL⁻¹: TVC, 7.0 ± 0.1 ; *Vibrio*, 5.4 ± 0.4 ; LAB, < 2.3). However, haemolytic vibrios ($4.3 \log \text{CFU mL}^{-1}$) were found in Remus-rotifers prepared 14 dph.

4.3.3 Larval survival, growth and development

In 2005, larvae fed treated rotifers (S5+6) had a low survival rate ($5.3 \pm 4.0\%$) at 27 dph compared to untreated larvae ($13.4 \pm 11.6\%$), though both groups showed a large variation between larval silos. Growth of larvae fed treated rotifers could not be evaluated because of the too few larvae left on the last sampling day. In 2006, the Remus-fed group showed about half of the average survival rate observed for the control groups (survival rate of $10.1 \pm 5.6\%$ vs $24.6 \pm 6.5\%$, respectively). On average, untreated larvae were 2.4 times (relative ratio, RR) as likely to survive compared to Remus-fed larvae. However, analysis of larval lysates for proteolytic activity and immunological proteins of importance in cod at early stage indicated a similar development for control and larvae fed treated rotifers (see Figure 5 and Figure 6). This was also reflected in similar larval length and dry weight though the latter parameter was in average about 16% lower in Remus-fed larvae than control ones (*Paper IV*).

4.4 Comparison of microbiota in treated and control rearing systems

This work aimed to study and identify the dominant culturable microbiota of cod rearing systems at pre- and posthatch stages and to characterise the effect of specific treatments during the 2004 and 2005 spawning seasons (*Paper III*). Cultivation methods were used to retrieve bacterial isolates and allow for their enumeration and characterisation, which could not be achieved by culture-independent methods. Current hatchery practices (ova disinfection and antibiotic treatment of unhealthy larvae) and specific putative probiotics identified earlier (*Paper I*) and applied to ova and larvae (*Paper II*) or rotifers were evaluated. Further, the

determination of virulence-related phenotypic traits among bacterial isolates was emphasised, including haemolysin, siderophores and AHLs, and their relation to larval survival evaluated. In total, 571 isolates were evaluated by phenotypic analysis which discerned 174 groups in 2004 (n=405) and 50 groups in 2005 (n=166). A representative selection of 257 isolates was done for partial 16S rRNA gene sequencing.

This analysis resulted in the identification of 75 OTUs belonging to five phyla. Thirty-seven of them were unique while 38 OTUs occurred more than once. Table 11 lists the 220 isolates distributed among these 38 OTUs and their incidence on each isolation year. γ -Proteobacteria was the largest phylum with *Pseudoalteromonas* spp. dominating as well as *V. splendidus* in 2005. Eighteen OTUs were common for both years. Table 12 provides an overview of the culturable microbiota isolated from newly fertilised ova, larvae, rearing water, supplements and feed in 2004. Table 13 illustrates the effect of microbial treatments on ova and larval microbiota composition, being compared to control groups. Microbiota composition is based on isolated MA colonies, proportionally relating to TVC given in the tables.

4.4.1 Microbiota at prehatch stage

Overall, a diversified culturable microbiota with prevailing γ -Proteobacteria was observed at the prehatch stage. In 2004, disinfection of newly fertilised ova with glutaraldehyde was efficient towards the dominating *Marinomonas* sp., leading to a 1000-fold reduction in TVC, while *Pseudoalteromonas* sp. survived the treatment to some extent Table 12. These two genera were also found among the rearing water isolates (data not shown). In 2005, glutaraldehyde disinfection of ova (1 dpf) led to an immediate, almost 100-fold reduction of ova microbial load followed by rapid microbial growth (Table 13). Ova disinfection was apparently not as efficient as in 2004 or recontamination of ova occurred since a more diversified microbiota was observed (*Paper III*).

Table 11. Phyla and defined OTUs of bacterial isolates (n=220) from cod rearing in 2004 and 2005 based on partial 16S rRNA gene sequencing and a NCBI Blast alignment search

Phylum*	OTU code †	Closest match in GenBank (% similarity)	Accession number	Number of isolates from 2004	Number of isolates from 2005
Bacteroidetes (611-682 bp)	Ba-C1	<i>Joostella marina</i> En5 (99) or <i>Flavobacterium</i> sp. R564 (99)	EF660761	1	2
	Ba-C2	<i>Tenacibaculum ovoliticum</i> SE1 (99)	EF474152		2
	Ba-C3	<i>Lacinutrix</i> sp. MEBiC01653 (99)	AY771741		10
	Ba-C4	<i>Psychroserpens burtonensis</i> S3-13 (99)	EU581705	2	1
Actinobacteria (694-887 bp)	Ac-C1	<i>Micrococcus luteus</i> AUH1 (99)	AY771723	3	
	Ac-C2	<i>Arthrobacter bergerei</i> (100)	EF187229	3	
	Ac-C3	<i>Microbacterium oxydans</i> L2 (98)	AJ609631	4	4
	Fi-C1	<i>Enterococcus thailandicus</i> 933 (99)	GQ923776	5	1
Firmicutes (535-993 bp)	Fi-C2	<i>Enterococcus phoeniculicola</i> (98)	EF197994	3	4
	Fi-C3	<i>Enterococcus pseudovarium</i> ATCC 49372 (100) or <i>Enterococcus devriesei</i> LMG 14595 (100)	AY028437	1	2
	Fi-C4	<i>Vagococcus fluvialis</i> IFS3 (100)	DQ411809	3	
	Fi-C6	<i>Alkalibacterium putridaligicola</i> (99)	AJ891167	5	
Gamma (γ) – Proteobacteria (425-1100 bp)	Fi-C7	<i>Alkalibacterium olivoapovlitticus</i> WW2-SN4a (99)	AB272361	3	
	Fi-C9	<i>Exiguobacterium</i> AT1b (99)	AB294170	3	
	GP-C1	Uncultured bacterium clone 4753K9-26 (98)	AF143511	3	
	GP-C2	<i>Enterobacter</i> sp. TP2MC (99)	CP001615	2	
	GP-C3	Uncultured bacterium clone 1C226626 (99)	FJ041853	2	
	GP-C4	<i>Vibrio</i> sp. FALF296 (100)	GU272376	4	
		<i>V. gallicus</i> or <i>V. hepatarius</i> or <i>V. xuii</i> (99)	EU799070		3
		<i>Pseudomonas stutzeri</i> ST27MN2 (100)	EU655384	3	5
	GP-C5	Marine arctic deep-sea bacterium FC10 (99)	AJ633560	1	2
	GP-C6	<i>Serratia fonticola</i> UTAD54 (100)	AJ557844		3
	GP-C7	<i>Pseudoalteromonas</i> sp. BSW20607 (100)	AY236502	4	
	GP-C8	<i>Vibrio splendidus</i> (100)	EF639379	37	16
	GP-C9	<i>Vibrio proteolyticus</i> A2G-5B (100)	AJ874364	8	15
	GP-C10		DQ995521	7	

Alpha (α) – Proteobacteria (425-1100 bp)	GP-C11	<i>Vibrio aestuarianus</i> (99)	AJ845016	2	
	GP-C12	<i>Vibrio natriegens</i> UST040801-014 (100) or <i>Vibrio alginolyticus</i> UST950701-007 (100)	EU834012	3	2
	GP-C13	<i>Colwellia</i> sp. BSw20188 (99)	EU833995	1	7
	GP-C14	<i>Colwellia</i> sp. EM0397 (99)	EU365586	3	1
	GP-C15	<i>Psychrobacter</i> sp. BSw20884b (100)	FJ392832	5	
	GP-C17	<i>Pseudoalteromonas</i> sp. BSw10502 (99)	GU166132	2	1
	GP-C19	<i>Halomonas</i> sp. DG1230 (100)	EF375570	1	2
	GP-C20	<i>Marinomonas</i> sp. M12-1 (98)	DQ486477	3	
	GP-C22	<i>Pseudomonas</i> sp. BCw159 (100)	FN377704	3	
	AP-C1	<i>Ruegeria</i> sp. S3940 (100)	FJ889609	3	
	AP-C2	<i>Roseovarius nubinhibens</i> DSM15170 (100)	FJ460046	2	
	AP-C3	<i>Martella</i> sp. AD-3 (99)	DQ915625	1	1
	AP-C4	<i>Phaeobacter</i> sp. NH52F (100)	GU086202	1	1
	AP-C5	<i>Sulfitobacter</i> sp. KS8-4 (99)	FJ232451	2	
			FJ889529	2	2
			Total	133	87

* Range of OTU sequence length (bp, base pairs) used for Blast alignment search given in parenthesis for each phylum

† OTU code defined for sequenced isolates identified more than once.

Table 12. Microbiota in cod rearing systems in 2004. Bacterial composition (# isolates per sample=20) expressed as percentage (%) of total culturable microbiota (TVC) on marine agar.

OTU code	Sample type	Ova 1 dpf		Supplement		28-day RW		28-day larvae		56-day larvae		Feed d56
		TVC (log CFU g ⁻¹ or mL ⁻¹)	<i>Vibrio</i> count (log CFU g ⁻¹ or mL ⁻¹)	Algae	E-Art	Tank 1	Tank 2	Tank 1	Tank 2	Tank 1	Tank 2	
Ac-C1	Closest match in GenBank (% similarity)	Untr	Disinf									
	<i>Micrococcus luteus</i> (99)											5
	<i>M. luteus</i> (99) ^a							5	5			
Ac-C2	<i>Arthrobacter bergerei</i> (100)								5			
	<i>Arthrobacter</i> sp. (99) ^b											10
Ac-C3	<i>Microbacterium oxydans</i> (98)					5					5	
Fi-C1	<i>Enterococcus thailandicus</i> (99)											5
Fi-C2	<i>Enterococcus phoeniculicola</i> (98)					30						
Fi-C3	<i>Enterococcus pseudoavium</i> (100)					5			5			10
Fi-C4	<i>Vagococcus fluvialis</i> (100)											
	<i>Lactococcus lactis</i> ssp. <i>lactis</i> (99) ^c							30	5	5	5	5
Fi-C6	<i>Alkalibacterium putridalgalicola</i> (99)					25						
Fi-C7	<i>Alkalibacterium olivoapovliticus</i> (99)					10						
Fi-C9	<i>Exiguobacterium</i> sp. (99)					5		C				
	<i>Planococcus citreus</i> (99) ^d											5
	<i>Macrococcus caseolyticus</i> (99) ^e											10
	<i>Sporosarcina</i> sp. (98) ^f											5
	<i>Staphylococcus sap. ssp bovis</i> (99) ^g											
	<i>Bacillus thuringiensis</i> (98) ^h						15		5			
Ba-C3	<i>Lacinutrix</i> sp. (98)										15	
Ba-C4	<i>Psychroserpens burtonensis</i> (99)							5		65	5	
	<i>Bizionia saleffrena</i> strain (97) ⁱ								5			
AP-C2	<i>Roseovarius nubinhibens</i> (100)					5						
AP-C4	<i>Phaeobacter</i> sp. NH52F (100)						5				10	
	Alpha proteobacterium (100) ^j					5						

Table 13. Microbiota in cod rearing systems differently treated in 2005. Bacterial composition (# isolates per sample=10) expressed as percentage (%) of total culturable microbiota (TVC) on MA. Larval survival rate (%) for each silo at last experiment day.

Sample type		Ova		Ova		Larvae		Control		Bathed		Rot*		Treated	
		1 dpf	2.1	11 dpf	5.2	2 dph	>6	27 dph	6.6	27 dph	6.5	6.4	6.2	6.4	6.5
TVC (log CFU g ⁻¹ or mL ⁻¹)		3.7	2.1	5.9	5.2	5.3	6.6	6.6	6.6	6.5	6.4	6.4	6.2	6.4	6.5
Vibrio counts (log CFU g ⁻¹ or mL ⁻¹)		<1.3	1.9	<3	3.3	2.6	5.4	3.9	4.7	4.2	4.2	4.9	5.2	5.2	6.3
Larval survival rate (%)							21.6 ^b	5.2 ^d	42.1 ^a	11.2 ^c					5.3 ^{d†}
OTU code	Closest match in GenBank (% similarity)	SID	AHL	Untr	Dis	Co	B	Co	B	S3	S4	S1	S2	Rot	T- Rot
Ac-C2	<i>Arthrobacter bergerei</i> (100)	+	nd									20	20		10
Ac-C3	<i>Microbacterium oxydans</i> (98)	-	nd							10			20		
	<i>Microbacterium</i> sp. (95) ¹	-	nd											10	
Fi-C1	<i>Enterococcus thailandicus</i> (99)	-	nd												
	<i>Joostella marina</i> or														
Ba-C1	<i>Flavobacterium</i> sp. (99)	-	-						20	20			20		
Ba-C2	<i>Tenacibaculum ovolyticum</i> (99)	-	na												
Ba-C3	<i>Lacinutrix</i> sp. (98)	-	+/-						40	60		50	10	30	30
Ba-C4	<i>Psychroserpens burtonensis</i> (99)	+	-												
AP-C2	<i>Roseovarius nubinhibens</i> (100)	-	na												
AP-C3	<i>Marteella</i> sp. (99)	-	na										10		
AP-C5	<i>Sulfitobacter</i> sp. (99)	+/-	nd						10	10					
	<i>Sulfitobacter</i> sp. or														
	<i>Staleyia</i> sp. (100) ²	+	-						10						
	<i>Sulfitobacter</i> sp. (99) ³	+	-						10						
	Arctic sea ice bacterium (100) ⁴	-	-		10										
GP-C3	Uncultured bacterial clone (99)	-	-		10	10	10								
GP-C4	<i>Vibrio</i> sp. (100)	+++	+											20	20
GP-C6	Marine arctic deep sea bact.(99)	+/-	-												
GP-C8	<i>Pseudalteromonas</i> sp. (100)	+	+/-						40	50	30	10	20	10	10
GP-C9	<i>V. splendidus</i> (100)	++	+/-						T		T	20			50

GP-C13	<i>Colwellia</i> sp. (99)	+/-	-	30	30	20	40	10
GP-C14	<i>Colwellia</i> sp. (99)	-	-			10		
GP-C17	<i>Pseudalteromonas</i> sp. (99)	+	nd	10				
GP-C19	<i>Halomonas</i> sp. (100)	++	+		20			
	<i>Alcanivorax</i> sp. (90) ⁵	+	-	10				
	<i>Psychrobact. okhotskensis</i> (99) ⁶	++	-	10				
	<i>Psychrobacter nivimaris</i> or <i>Psychrobacter glacincola</i> (99) ⁷	-	-	20				
	<i>Psychrobacter celer</i> (100) ⁸	+	-		10			10
	Gamma-proteobacterium (99) ⁹	+	-			10		20
	Gamma-proteobacterium (92) ¹⁰	-	-				10	
	Gamma-proteobacterium (96) ¹¹	-	na					10
	<i>Oleispira antarctica</i> (99) ¹²	-	-				10	
	<i>Vibrio</i> sp. (99) ¹³	-	-	20	40	20	10	30
	Lost isolates	-	-				40	10

dpf, days postfertilisation; dph, days posthatch; TVC, total viable counts on MA; Ac, Actinobacteria; Fi, Firmicutes; Ba, Bacteroidetes; AP, Alpha-Proteobacteria; GP, Gamma-Proteobacteria; SID, siderophore production (none (-) to very much (+++)); AHL, production of acylated homoserine lactones based on either CV026 or pZLR4 monitor strain; Untr, untreated; Dis, disinfected by glutaraldehyde; Co, control group; B, bathed group; SI, larvae from silo 1, etc; S5+6, larvae pooled from silos 5 and 6; T-Rot, treated enriched rotifers; Rot, enriched rotifers; sampled after rinsing (20 dph); [†] average survival rate for S5 (8.1%) and S6 (2.5%) at 27 dph; nd, not determined; na, not available; N, isolated from NAP medium; T, isolated from TCBS medium. Bacterial details (Genbank accession number): ¹ *Microbacterium* sp. E920 (AY368525); ² *Sulfitobacter* sp. KS8-4 (FJ889529) or *Staleyia* sp. LM-10 (AJ534235); ³ *Sulfitobacter* sp. ARCTIC-P49 (AY573043); ⁴ Arctic sea ice bacterium ARK9996 (AF468381); ⁵ *Alcanivorax* sp. S2-5 (FJ218426); ⁶ *Psychrobacter okhotskensis* MD17 (NR024806); ⁷ *Psychrobacter nivimaris* KOPRI24925 (EF101544) or *Psychrobacter glacincola* ANT9104 (AY167281); ⁸ *Psychrobacter celer* 32 (FJ613610); ⁹ Gamma-proteobacterium UMB6E (AF505730); ¹⁰ Uncultured Gamma-proteobacterium Y134 (EU328090); ¹¹ Uncultured Gamma-proteobacterium (AF466900); ¹² *Oleispira antarctica* RB-8 (NR025522); ¹³ *Vibrio* sp. F74 (GU223580). Different letters (a,b,c,d) indicate significant ($P<0.05$) difference in survival rate among larval silos. Box with full line indicates that isolates in designated samples produced siderophores, and dashed line means that only some isolates did. AHL production of isolates detected using monitor strain pZLR4 (light gray box) or both pZLR4 and CV026 (dark gray box).

In 2006 (**Paper IV**), Pyceze[®] was used for ova disinfection at early (0 dpf) and late (10 dpf) stages. Microbiological evaluation of ova incubators on 3 dpf showed that the microbiota was slowly developing after disinfection, as undetectable or low microbial levels (TVC and *Vibrio* counts) were observed on ova and in rearing water (data not shown). A week later, a significant ($P<0.05$) increase in TVC was noticed for ova and rearing water, with high *Vibrio* levels on ova representing almost 10% of total culturable microbiota (Table 9). *Vibrio* levels in rearing water were significantly ($P<0.05$) lower than TVC. Based on replica plating on BA-NaCl, mostly all *Vibrio* colonies were found to be haemolytic (10 dpf), both for ova and rearing water (data not shown). The second Pyceze[®] treatment led to a slight decrease in the microbial load of rearing water, with no effect on ova.

In 2005 (**Paper III**), ova microbiota was dominated by γ -Proteobacteria as in 2004, but included several OTUs not seen in the earlier trial (Table 13). The effect of a bacterial bathing treatment on ova microbiota composition was assessed in 2005. At 11 dpf, only two (GP-C3 and GP-C13) of the OTUs initially present on ova were detected independently of treatments. However after three bacterial treatments, a different microbiota resulted on bathed ova compared to control ova. Seventy percent of the culturable microbiota of bathed ova constituted of *Pseudoalteromonas* and *Colwellia* spp., while that of control ova was heterogeneous. Presence of *V. splendidus* was confirmed from TCBS medium on control ova prior to hatching.

4.4.2 Microbiota at posthatch stage

Posthatching, a multiphyla microbiota developed influenced by the treatments and diets applied (**Paper III**). In 2005, microbiota of newly hatched larvae showed that two bacterial genera, *Pseudoalteromonas* and *Colwellia*, were prevailing as similarly found on ova prior to hatching (Table 13). Presence of *V. splendidus* was confirmed from TCBS medium on newly hatched larvae (not surface disinfected) and their rearing water (2 dph). *Aliivibrio salmonicida* (99% similarity to Genbank EU091324) was also isolated from the control rearing water (TCBS, data not shown). Strain 04-394 used for bathing was confirmed from NAP medium on 2-dph larvae hatched from bathed ova. Common OTUs found in control (S3-S4) and bathed (S1-S2) larvae 27 dph included Ac-C3, Ba-C1, Ba-C3 and GP-C8, all common to rotifers. Bathed larvae harboured two (S1) or one (S2) of the bacteria used for bathing, strains 04-394 (Fi-C1) and/or 04-279 (Ac-C2). Larvae fed treated rotifers (S5+6) fared poorly 20

dph, with high *Vibrio* counts in agreement with high MA levels of GP-C9 and GP-C4, one of the OTUs found in treated rotifers. Bacteroidetes bacteria were present at high levels (30-80%) in larvae, except in those fed treated rotifers (Table 13).

Microbiota analysis of rotifers fed to larvae 20 dph revealed that OTUs Ba-C3 and GP-C8 were common to untreated and treated rotifers, while γ -Proteobacteria dominated in treated rotifers (Table 13). Untreated rotifers also contained *Microbacterium* sp. and *Roseovarius nubinhibens*, but high occurrence of lost isolates (40%) gives a less clear picture. Yeasts and LAB occurred naturally in untreated rotifers but at lower levels than in treated rotifers. Rotifers analysed in 2004 revealed *V. proteolyticus*, *Rva. nubinhibens*, *Microbacterium oxydans*, *Joostella marina/Flavobacterium* sp., *Pseudoalteromonas*, *Enterobacter* and *Ruegeria* spp. (data not shown).

The microbiota of commercial larval rearing units was also studied (Table 12). At posthatch stage (28 dph), γ -Proteobacteria dominated among the rearing water microbiota while larval microbiota was heterogeneous. *Pseudoalteromonas* and *Vibrio* (GP-C10, GP-C12) spp. found in enriched *Artemia* were present in rearing water and 28-dph larvae (except for GP-C12). *Vibrio* spp. (GP-C9, GP-C12), known to be haemolytic, were detected in the rearing water of silos (data not shown, GP-C9 only) and tanks. GP-C9 was also isolated at high levels from larvae in tank 1. Algal concentrate and dry feed mostly contained Gram-positive bacteria, dominated by Firmicutes. Both had little or no apparent effect on the tank microbiota composition. On day 55 ph, larvae from tank 1 showed unhealthy behaviour and were treated with interspectin. Lower TVC and *Vibrio* counts were detected in larvae 56 than 28 dph, and changes were observed in microbiota composition. The microbiota of interspectin-treated larvae was homogeneous, dominated by *Psychroserpens burtonensis* and *Pseudomonas stutzeri*. Non-treated larvae (tank 2) had lower levels of γ -Proteobacteria compared to 28 dph because of increased importance of Bacteroidetes and α -Proteobacteria; however, 45% of isolates were lost (died before identification). Several γ -Proteobacteria grew on CFC medium, namely *Pseudomonas*, *Pseudoalteromonas*, *Vibrio* and *Marinomonas* spp., as well as Enterobacteriaceae and Firmicutes. Similarly, TCBS agar was not totally selective as species belonging to other genera than *Vibrio* were isolated, including *Marinomonas*, *Pseudomonas* and *Pseudoalteromonas* spp.

4.4.3 Differentiation within OTUs by API tests

Additional biochemical tests, API 20 E and API 20 NE, were used to confirm the phenotypic heterogeneity observed within some defined OTUs. Table 14 lists the 8 OTUs evaluated, API subtypes obtained, differentiating tests and origin of isolates tested. The most heterogeneous OTUs included *Pseudoalteromonas* sp. (GP-C8), *V. splendidus* (GP-C9) and *Colwellia* sp. (GP-C13). *Lacinutrix* sp. (Ba-C3) had two subtypes while *J. marina/Flavobacterium* sp. (Ba-C1), *Vibrio* sp. (GP-C4) and *V. natriegens/V. alginolyticus* (GP-C12) were homogeneous OTUs.

Table 14. Differentiation of isolates within defined OTUs using API biochemical tests. Resulting API subtypes and origin of isolates given. API 20 NE for non-fermentative bacteria (n=52) and API 20 E for those fermentative (n=30).

API test type		OTU code *	API subtype (N _{isolates}) and differentiating tests	Origin of isolates
API 20 NE		Ba-C1	identical (2)	larvae S2-S4
		Ba-C3	a (12): gelatinase positive	a: rotifers, treated rotifers, larvae S1-S2-S3-S4
		GP-C8	b (2): gelatinase negative	b: larvae S1-S3
			a (2) } differed by 9 tests	a: larvae S1-S3
			b (3) }	b: treated rotifers, larvae S2-S3
			c (3) }	c: bathed ova, larvae S1-S3
		d (2) }	d: larvae S1-S3	
		single (11): differed by 14 tests	single: rotifers, bathed ova, larvae S1 (set as “e”), S3 and S5+6 (set as “f”)	
API 20 E		GP-C13	a (10): β-galactosidase positive	a: control ova, bathed ova, larvae S1-S3
			b (2): β-galactosidase negative	b: newly fertilised ova
			single (1): β-galactosidase positive and 4 differing tests	single: newly fertilised ova
		GP-C19	single (2): differed in phenylacetic and capric acid assimilation and indole formation	disinfected ova
		GP-C4	identical (5)	treated rotifers, larvae S4 and S5+6
		GP-C9	a (11): arginine dihydrolase (ADH) negative	a: control ova, S1-rearing water, larvae S1-S3-S5+6
			b (8): ADH positive	b: control ova, rearing water (from bathed incubator, S1 and S3), larvae S3-S5+6
		single (2): ADH/gelatinase negative, β-galactosidase positive or negative	single: S3 rearing water	
		GP-C12	identical (4)	enriched <i>Artemia</i>

* Bacterial identification details for operational taxonomic units (OTU) codes are given in Table 11.

4.4.4 Siderophores, haemolysin and AHL production among microbiota

Production of siderophores and detection of AHL signals for several isolates are presented in Table 13. A total of 20 isolates from eight OTUs produced AHLs, with strains from three OTUs (GP-C4, GP-C9, GP-C19) inducing violacein and β -galactosidase production of the monitor strains, *C. violaceum* (CV026) and *Ag. tumefaciens* NT1 (pZLR4), respectively. AHL-producing OTUs not shown in Table 13 are GP-C5 and GP-C12 from enriched *Artemia* and *Aliivibrio salmonicida* from rearing water (S3). Members of seven OTUs (GP-C4, GP-C5, GP-C8, GP-C9, GP-C12, GP-C19 and *Aliivibrio salmonicida*) produced both siderophores and AHLs. Among the isolates obtained in 2005, a strong haemolytic response was observed in *Aliivibrio salmonicida* and *V. splendidus* strains, while a *Vibrio* sp. (GP-C12) responded to a lesser extent. Overall data is provided in Table 15.

4.4.5 Effects of microbiota on larval survival

At the end of the 2005 trial, the performance (survival rate) of each larval silo was evaluated (Table 13) and found to differ significantly within and between treatments ($P < 0.05$). A multivariate comparison of larval survival rate and occurrence of OTUs detected in corresponding larval samples (S1 to S6) was conducted. The heterogeneous OTUs were subdivided based on the API subtypes obtained (e.g. Ba-C3a and Ba-C3b, GP-C8a-b-c-d and two single subtypes assigned as GP-C8e and GP-C8f, etc). Ba-C3und (undetermined) was assigned to strains not assayed by API test. Principle Component Analysis (PCA) revealed that 13 of the designated bacterial groups ($n=18$) were of importance in explaining the variation (79%) observed between silos. Figure 11 presents the PCA biplot for these bacterial groups and larval survival rate of the silos. Larval survival rate influences both principle components, PC1 ($r = -0.525$) and PC2 ($r = 0.837$). Therefore, samples with high survival rates are positioned in the upper left quadrant (S1), in contrast to low survival silos heading downwards (S2 to S4) or to the right (S5+6). Similarly, OTUs highly correlated to survival are found in this upper left position, while those related to lower survival rates lie in opposite positions. Clustering observed on this plot with respect to larval performance allowed for the presumptive determination of beneficial or harmless (“good”) bacterial groups, those possibly with negative effects and two detrimental/opportunistic (“bad”) bacterial clusters, one relating to S2- and S4-larvae and the other to S5+6 larvae.

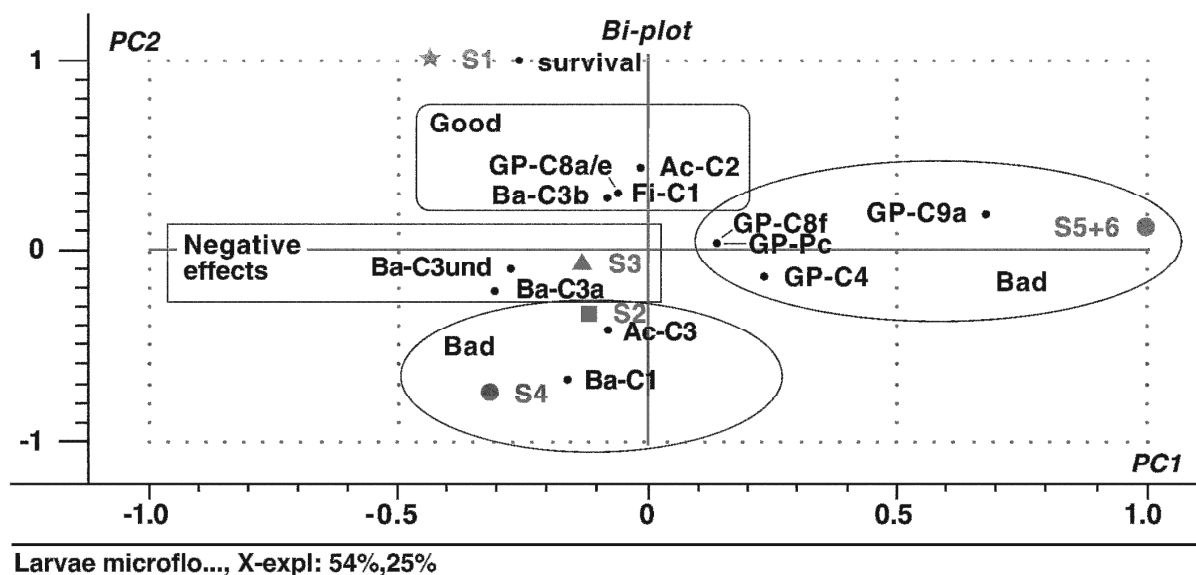


Figure 11. PCA biplot relating larval survival rate and occurrence (%) of genotypic groups (OTUs) in corresponding larval samples from 2005

Samples: S1 and S2 are bathed larvae, S3 and S4 are untreated larvae (control), S5+6 are larvae fed treated rotifers. Sample symbols indicate the range categories of survival rates (2.8 to 42.1%). Genotypic groups are clustered as follows: good (Fi-C1, GP-C8a, GP-C8e, Ba-C3b and Ac-C2), some negative effects (Ba-C3a and Ba-C3und) and bad (Ac-C3 and Ba-C1; GP-C8f, GP-Pc, GP-C9a, GP-C4).

Table 15. Genotypic, phenotypic and origin data of representative bacterial strains tested for putative virulence factors

OTU code *	Closest match in Genbank (% similarity)	Accession number	Strain code	API subtype	QS response CV026	pZLR4	Siderophore production	Haemolytic reaction	Strain origin
(AP)	<i>Staleya</i> sp. (100) or <i>Sulfitobacter</i> sp. (100)	AJ534235 FJ889529	172		-	-	+	-	d11-control ova
	Arctic sea ice bacterium (100)	AF468381	143		-	-	-	-	d1-disinfected ova
	<i>Sulfitobacter</i> sp. (99)	AY573043	171		-	-	+	-	d11-control ova
Ba-C1	<i>Joostella marina</i> (99) or <i>Flavobacterium</i> sp. (99)	EF660761 EF474152	12		-	-	-	-	d27-larvae (S2)
Ba-C3	<i>Lacinutrix</i> sp. (98)	EU581705	32	a	-	+	-	-	d27-larvae (S4)
			30	a	-	+	-	-	d27-larvae (S3)
			101	a	-	-	-	-	d20-control rotifers
			103	a	-	-	-	-	d20-control rotifers
			106	a	-	-	-	-	d20-PROB rotifers
			109	a	-	-	-	-	d20-PROB rotifers
			27	a	-	-	-	-	d27-larvae (S3)
			4	b	-	-	-	-	d27-larvae (S1)
			29	b	-	-	-	-	d27-larvae (S3)
Ba-C4	<i>Psychroserpens burtonensis</i> (99)	AY771723	188		-	-	+	-	d2-larvae (S1)
(GP)	<i>Alcanivorax</i> sp. (90)	FJ218426	134		-	-	+	-	d1-fertilised ova
	<i>Psychrobacter okhotskensis</i> (99)	NR024806	138		-	-	++	-	d1-fertilised ova
	<i>Psychrobacter celer</i> (99)	FJ613610	78		-	-	+	-	d20-larvae (S5+6)
	<i>Psychrobacter nivimaris</i> (99)	EF101544							
	<i>Psychrobacter glacincola</i> (99)	AY167281	149		-	-	-	-	d1-disinfected ova
GP-C4	<i>Aliivibrio salmonicida</i> (100)	EU091324	184		-	+	++	++	d2-seawater (S3)
	<i>Vibrio</i> sp. (100)	EU655384	73		+	+	+++	-	d20-larvae (S5+6)
			108		+	+	+++	-	d20-PROB rotifers
			40		+	+	+++	-	d27-larvae (S4)

Table 15. Genotypic, phenotypic and origin data of representative bacterial strains tested for putative virulence factors (continued)

OTU code *	Closest match in Genbank (% similarity)	Accession number	Strain code	API subtype	QS response CV026	QS response pZLR4	Siderophore production	Haemolytic reaction	Strain origin
GP-C5	<i>Pseudomonas stutzeri</i> (100)	AJ633560	63		-	+	++	-	enriched <i>Artemia</i>
			91		-	-	+	-	<i>Artemia</i> nauplii
GP-C8	<i>Pseudoalteromonas</i> sp.(100)	EF639379	85	single	-	-	+++	-	d20-larvae (S5+6)
			7	a	-	-	-	-	d27-larvae (S1)
			162	c	-	-	+	-	d11-bathed ova
			189	c	-	-	+	-	d2-larvae (S1)
			201	d	-	-	+	-	d2-larvae (S3)
			110	b	-	-	+++	-	d20-PROB rotifers
			75	f	-	-	++	-	d20-larvae (S5+6)
			210	single	-	-	+	-	d2-larvae (S3)
			211	single	-	-	+	-	d2-larvae (S3)
			204	single	-	-	+	-	d2-larvae (S3)
			165	single	-	-	+	-	d11-bathed ova
			10	e	-	+	+++	-	d27-larvae (S1)
			25	a	-	-	+++	++	d27-larvae (S3)
			175	a	-	+	++	++	d11-control ova
			180	a	-	-	+	++	d2-seawater (S1)
			72	a	-	+	+++	++	d20-larvae (S5+6)
GP-C9	<i>Vibrio splendidus</i> (100)	AJ874364	195	a	-	-	+++	++	d2-larvae (S1)
			178	b	-	-	+	++	d11-ova seawater
			183	b	-	-	++	++	d2-seawater (S3)
			181	b	-	-	+++	++	d2-seawater (S3)
			174	b	-	+	++	++	d11-control ova
			81	b	-	+	++	++	d20-larvae (S5+6)
			22	b	+	+	+	++	d27-larvae (S3)
			179	b	+	+	+++	++	d2-seawater (S1)
			182	single	+	+	+++	++	d2-seawater (S3)

GP-C9	<i>V. splendidus</i> (100)	AJ874364	185	single	+	+	+++	++	d2-seawater (S3)
GP-C12	<i>V. natriegens</i> (100) or <i>V. alginolyticus</i> (100)	EU834012 EU833995	62		-	+	+++	+	enriched <i>Artemia</i>
GP-C13	<i>Colwellia</i> sp. (99)	EU365586	64		-	+	+++	+	enriched <i>Artemia</i>
			139	b	-	-	-	-	d1-fertilised ova
			141	b	-	-	-	-	d1-fertilised ova
			173	a	-	-	+	-	d11-control ova
			191	a	-	-	+	-	d2-larvae (S1)
			200	a	-	-	-	-	d2-larvae (S3)
GP-C19	<i>Halomonas</i> sp. (100)	DQ486477	148	single	+	-	++	-	d1-disinfected ova
			151	single	+	+	+	-	d1-disinfected ova

* OTU code defined for sequenced isolates identified more than once. Quorum sensing response given by *Chromobacterium violaceum* (CV026) and *Agrobacterium tumefaciens* NT1 (pZLR4). AP, alpha-Proteobacteria; Ba, Bacteroidetes; GP, gamma-Proteobacteria. Magnitude of siderophore production from none (-) to very much (+++). Magnitude of haemolytic reaction from none (-), some (+) to much (++).

4.5 Validation of two prospective probionts at cod juvenile stage

An intervention study was conducted at an early juvenile stage of cod rearing to confirm the properties of two prospective probionts, *Arthrobacter bergerei* (04-394) and *Enterococcus thailandicus* (04-279) isolated from a cod hatchery (**Paper I**). Juveniles (10 g) were fed expanded dry feed for 28 or 55 days, either untreated or treated with the probiotic bacteria in mixture or singly (10^{7-9} CFU g⁻¹ feed). Viability of probionts supplemented to the feed was evaluated during a two-month storage at -20, 4 and 15 °C and sensory evaluation of the probiotic feed performed at the end of storage. Juvenile growth, survival, feed conversion ratio, and microbial load of rearing water, juvenile gills and gut were examined (**Paper V**).

4.5.1 Viability and quality evaluation of probiotic dry feed

Viability assessment of probionts added to the feed stored at -20, 4 and 15 °C for up to 8 weeks showed that overall their initial inoculation level was maintained at all temperatures throughout storage (Figure 12), with least deviations observed at 15 °C for both probionts. After 8-week storage, little or no odour difference was found between the probiotic and control feeds, but the probiotic feed had a slightly darker colour (data not shown). The feed stored at -20 °C had a hint of oxidative off-odour, for both probiotic and control feeds. Overall, microbiological stability of the probionts added to the feed was demonstrated at all temperatures. Moisture content of probiotic feed after 2-week storage at -20, 4 and 15 °C was on average $6.0 \pm 0.4\%$ compared to $5.4 \pm 0.4\%$ in untreated feed. Product description claims a moisture content of 7%. A higher moisture content ($8.8 \pm 0.4\%$) was measured in another batch of feed used for the juvenile trials, as well as in the resulting probiotic feeds, $9.1 \pm 0.4\%$ (A), $8.4 \pm 0.4\%$ (B), $8.5 \pm 0.4\%$ (C).

During the juvenile feeding trial, evaluation of the microbiological quality of the expanded dry feed used revealed the presence of naturally occurring LAB (3.2 ± 0.0 log CFU g⁻¹) among the total culturable microbiota (TVC = 3.6 ± 0.1 log CFU g⁻¹) in the control diet. Initial concentration (log CFU g⁻¹) of added bacteria in the prepared probiotic feeds amounted to 7.3 ± 0.2 and 7.2 ± 0.2 for strain 04-394 in feeds A and C, respectively, but 8.7 ± 0.2 and 8.7 ± 0.0 for strain 04-279 in feeds A and B, respectively. After 4-week storage, levels of 04-279 had decreased significantly in feeds A (0.8 log) and B (0.6-0.7 log) ($P < 0.05$).

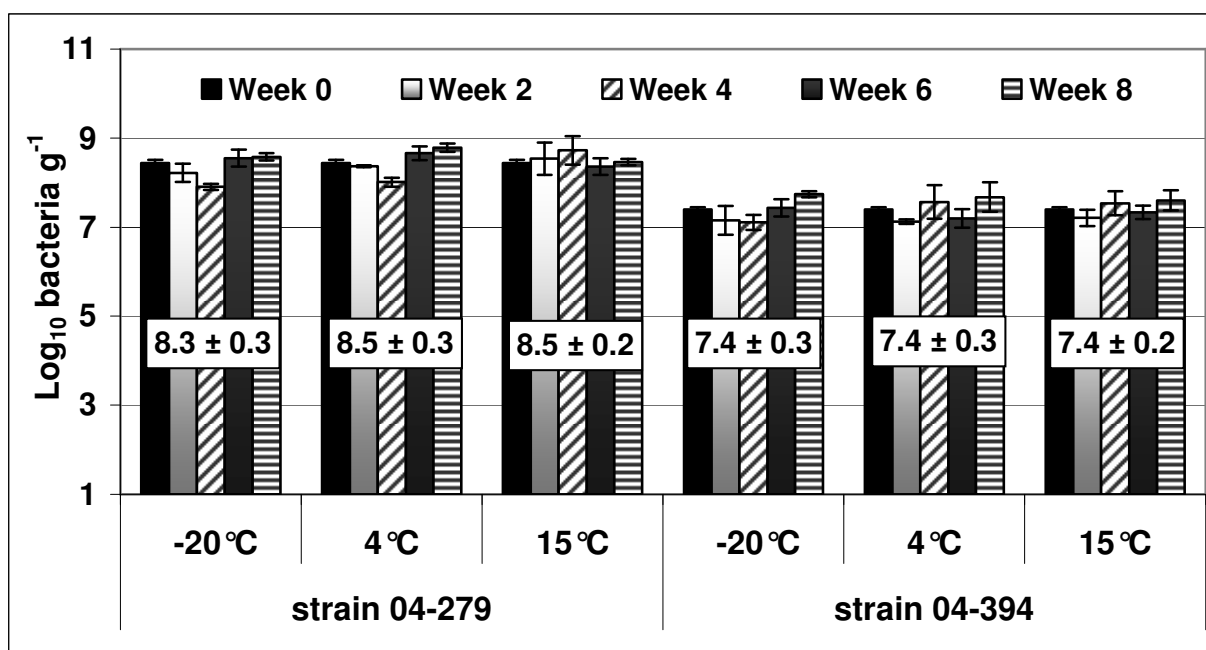


Figure 12. Viability of two probionts [*Arthrobacter bergerei* (04-279) and *Enterococcus thailandicus* (04-394)] on coated feed during 8-week storage at -20, 4 and 15 °C. Average probiont count (log CFU g⁻¹) and SD shown for each storage temperature over the whole storage period.

4.5.2 Effect of feeding treatments on microbial load of juveniles and rearing systems

Microbial load of the rearing water was assessed on days 0, 28 and 55. Initial TVC of the groups studied (control day 0) amounted to 3.1 ± 0.2 log CFU mL⁻¹ and did not increase significantly ($p > 0.05$) in tanks treated with probiotic feeds (Figure 13). TVC of the control rearing water increased significantly between days 28 (3.2 ± 0.3 log CFU mL⁻¹) and 55 (3.9 ± 0.0 log CFU mL⁻¹). Figure 13 illustrates the proportional composition of the culturable microbiota in relation to total culturable haemolytic counts in seawater at the beginning and the end of the experiment as well as on juvenile gills on day 55. The results demonstrate that the microbiological quality of the seawater was significantly improved by transferring the juveniles from their growing tanks to the study tanks, as evaluated 2 days after acclimatisation (day 0). This led to a decrease in levels of total culturable haemolytic bacteria, though statistically insignificant. These levels were generally maintained during the experiment, except for the C-fed tanks where the total culturable haemolytic counts of rearing water became significantly lower on day 55. Finally, initial *Vibrio* count of the rearing water was low (< 2.3 log CFU mL⁻¹), but decreased to lower levels in all groups as the trials progressed.

(data not shown). LAB counts were generally below the detection level and probionts were not detected (<10 CFU mL⁻¹) in the rearing water of treated tanks on days 28 and 55.

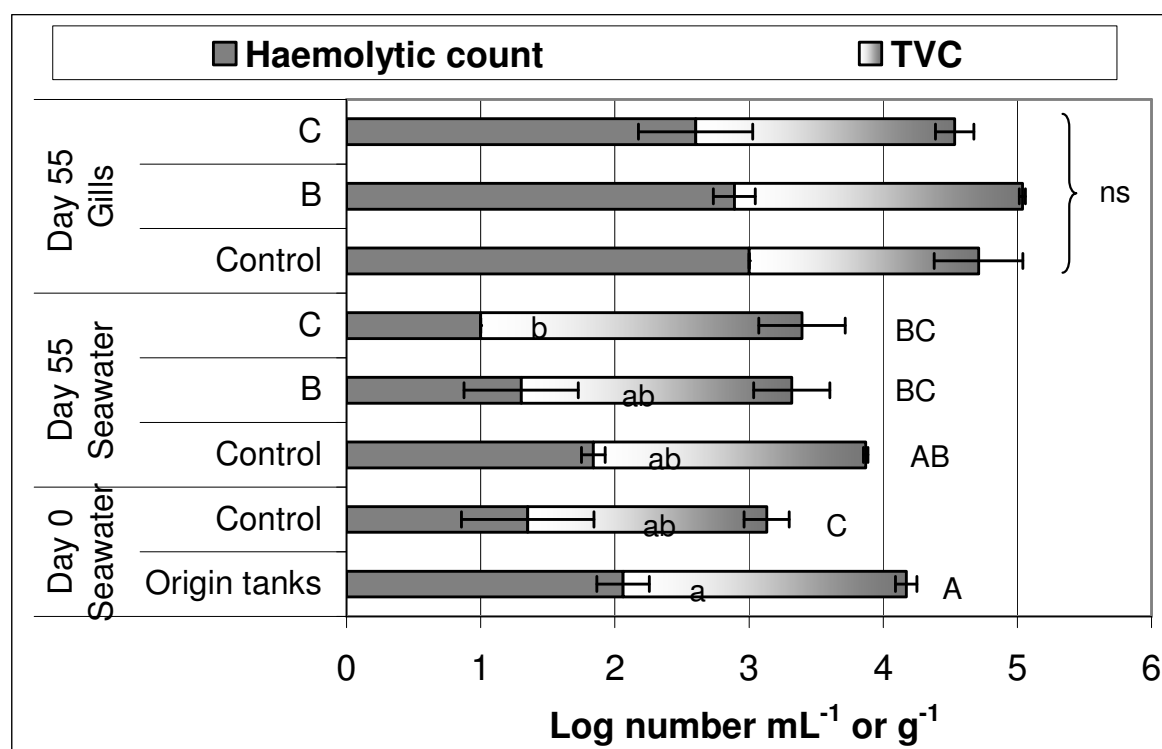


Figure 13. Proportional comparison of TVC and total culturable haemolytic counts in seawater initially (day 0) and on day 55, and on juvenile gills at the end of the experiment. Origin tanks, seawater counts from large scale tanks where juveniles were obtained. Treatments presented are control (regular feed), B (feed with probiont 04-279) and C (feed with probiont 04-394). Different letters indicate significant differences among treatments ($P<0.05$); ns, not significant. Seawater count below 2.3 log CFU mL⁻¹ is an estimate.

TVC and presumptive *Vibrio* counts of juvenile gills and gut at the end of the experiment are presented in Figure 14. Significant differences were not observed for TVC and *Vibrio* counts of gills among the differently treated juveniles ($P>0.05$). Interestingly, higher TVC and total culturable haemolytic counts were observed on juvenile gills than in rearing water on day 55 (Figure 13). Total culturable gut microbiota was significantly lower in A-fed juveniles compared to all other treatments ($P<0.05$). *Vibrio* counts were significantly lower in the gut of juveniles given feeds A and C, both containing strain 04-394, compared to control feed (Figure 14).

Probiotics were detected on the gills of juveniles given the probiotic feeds, constituting about 1% of the total culturable microbiota on days 28 and 55 (data not shown). On day 28,

strain 04-394 ($3.0 \pm 0.0 \log \text{CFU g}^{-1}$) accounted for over 10% of the gut microbiota of A-fed juveniles, but was not isolated ($<2.3 \log \text{CFU g}^{-1}$) on day 55 in C-fed juveniles. Strain 04-279 was not detected in the gut of A- and B-fed juveniles.

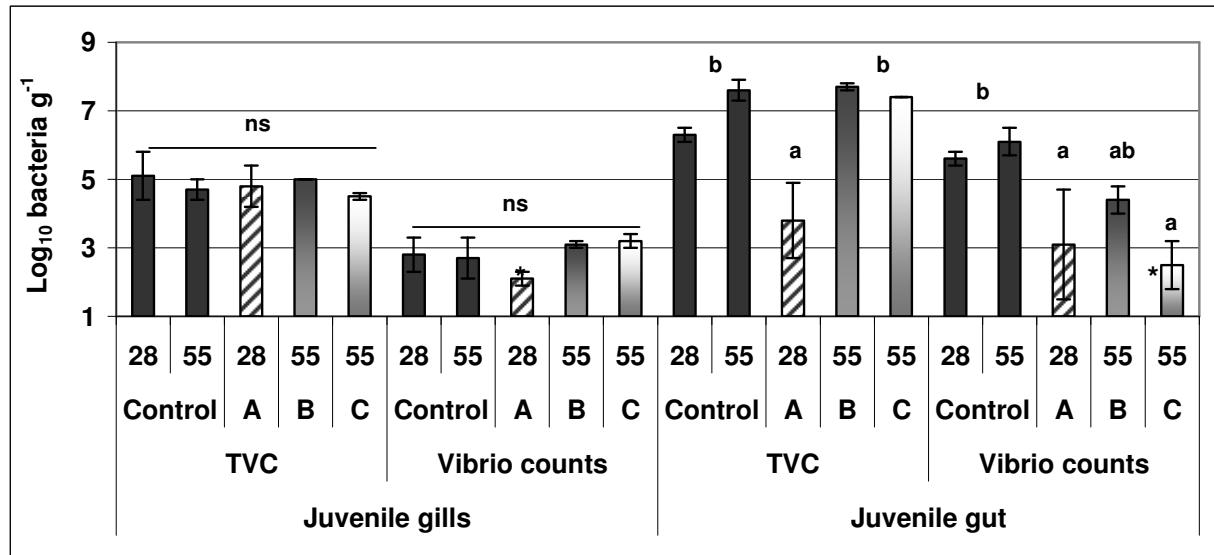


Figure 14. Effect of feeding treatments on microbiological load ($\log \text{CFU g}^{-1} \pm \text{SD}$) of juvenile gills and gut sampled on days 28 and 55
A, feed with both probionts; B, feed with 04-279; C, feed with 04-394. Different letters indicate significant differences among treatments ($P < 0.05$); ns, not significant; * count below $2.6 \log \text{CFU g}^{-1}$ is an estimate.

4.5.3 Juvenile survival, specific growth rate and feed conversion ratio

The effects of the feeding treatments and duration of trials on juvenile performance are shown in Table 16. SGR was significantly higher for C-fed (*Enterococcus* probiont) juveniles, with 38.2% faster growth compared to control juveniles after 28 days ($P < 0.05$). A- (both probionts) and B- (*Enterococcus* probiont) feeds also led to increased growth, namely 18.4% and 9.2% more than in control juveniles. In the latter period of trial II (d28-55), higher SGR were observed, with 2.9% and 7.2% faster growth for B- and C-fed juveniles, respectively, compared to the control group. FCR, calculated at the end of the trials, was highest in control juveniles but lowest in C-fed juveniles. Finally, survival rate was generally high for all treatments but found to be significantly higher ($P = 0.001$) for C-fed juveniles compared to the control group after 55 days.

Table 16. Effects of feeding treatments on juvenile performance

	Control	A-feed	B-feed	C-feed
SGR d0-28	0.76 ± 0.13 ^a	0.90 ± 0.15 ^{ab}	0.83 ± 0.09 ^{ab}	1.05 ± 0.07 ^b
SGR d28-55	1.38 ± 0.03 ^a	ND	1.42 ± 0.07 ^a	1.48 ± 0.10 ^a
FCR d0-28	2.83	2.43	ND	ND
FCR d0-55	2.05	ND	1.83	1.49
Survival d28	98.5 ± 1.4 ^a	99.6 ± 0.7 ^a	100 ± 0 ^a	100 ± 0 ^a
Survival d55	91.9 ± 6.2 ^a	ND	96.3 ± 1.8 ^{ab}	99.4 ± 0.9 ^b

Control, commercial feed; A-feed, supplemented with both probiotics; B-feed, supplemented with *Arthrobacter bergerei* (04-279); C-feed, supplemented with *Enterococcus thailandicus* (04-394). SGR, specific growth rate (% day⁻¹); FCR, feed conversion ratio; ND, not determined. Different letters within a row indicate significant differences among treatments ($P < 0.05$).

4.5.4 Overall impact of feeding treatments on juvenile performance and microbial status

Multivariate analysis of juvenile data obtained on days 28 and 55 considered the 7 parameters measured (TVC and *Vibrio* counts of gills and gut, survival rate, SGR and FCR). The first two principal components (PC1 and PC2) explained 75% (39% and 36 %, respectively) of the variation between the samples (data not shown). A better model was obtained by considering four variables, i.e. survival, SGR, FCR and gut *Vibrio* load as illustrated in Figure 15, where PC1 and PC2 explain 91% (54% and 37 %, respectively) of the variation between the four test groups. PC1 is described by high SGR from the right to high FCR to the left. Gut *Vibrio* count influences both PC1 and PC2, the latter being strongly influenced by the survival rate (upper part).

This bi-plot illustrates well the negative correlation found between SGR and FCR ($r = -0.912$) and between the survival rate and gut *Vibrio* counts ($r = -0.549$). A temporal effect was seen along PC1, with juveniles analysed on day 28 positioned towards the left part of the plot and those sampled on day 55 moving towards the right. This is explained by the higher FCR and lower SGR of the untreated (U1-U2) and treated (A1-A3) juveniles observed during the first month (trial I), as opposed to better growth and lower FCR seen in the 55-day trial (II) among untreated (U4-U5) and probiotic-fed (B1-B2 and C1-C2) juveniles. Overall, the higher survival and SGR, lower FCR and gut *Vibrio* load of probiotic-fed juveniles are demonstrated by the upper right position of these groups on the plot in contrast to the opposite position of controls.

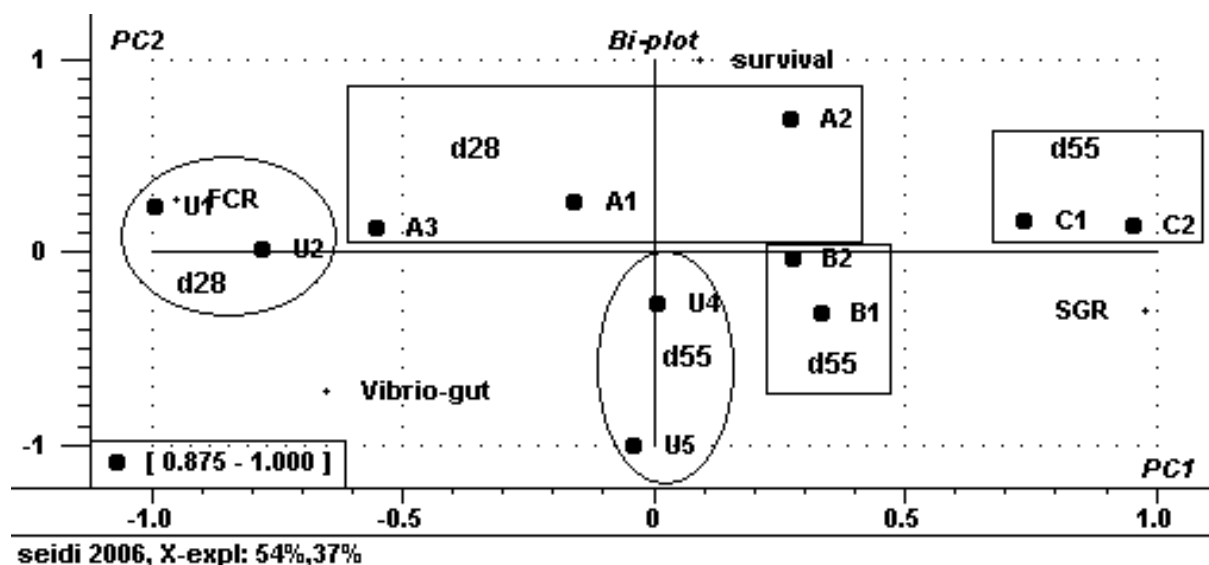


Figure 15. Effects of feeding treatments on juvenile performance and gut *Vibrio* load: scores (juvenile samples with dot) and loadings (variables measured) of a principle component analysis (PCA)

Samples: U1-U2 and U4-U5, untreated (control) juveniles analysed on days 28 and 55, respectively, A1-A3, juveniles fed mixed probiotic feed (A) for 28 days; B1-B2, juveniles fed *Arthrobacter*-probiotic feed (B) for 55 days; C1-C2, juveniles fed *Enterococcus*-probiotic feed (C) for 55 days. Variables: gut *Vibrio* counts (Vibrio-gut), specific growth rate (SGR), survival, feed conversion ratio (FCR).

5 DISCUSSION

The development of sustainable cod aquaculture is of high importance because of anticipated diminution in wild catches in the future. It is generally accepted that probiotics may contribute to increased competitiveness and success of aquaculture. In the first part of the present studies, the aquacultural environment of cod was screened for putative probiotic bacteria during pre- and posthatching periods in an experimental aquaculture station in Iceland. Five bacteria were selected for further analysis, which resulted in the selection of two prospective probionts. Their effects at different cod developmental stages on survival, growth, development and microbiota control were evaluated. Further, the dominant culturable microbiota of early cod rearing was characterised as influenced by specific treatments during two spawning seasons. Virulence-related phenotypic traits, including haemolysin, siderophores and AHLs, were studied among bacterial isolates and related to larval survival.

5.1 Screening for putative probiotic bacteria (*Paper I*)

The approach chosen was based on a set of criteria focusing on competitive exclusion of pathogens, active metabolite production, strain identification, growth characteristics and adhesion capacity of putative probionts. The inhibitory spectrum of representative isolates towards fish pathogens, a widely used characteristic for probiont determination, was verified. The selection of pathogens reflected the three main bacterial diseases encountered in cod aquaculture that are relevant to Icelandic rearing conditions: vibriosis (*V. anguillarum*); atypical furunculosis (*Aeromonas salmonicida* ssp. *achromogenes*); and coldwater vibriosis (*Aliivibrio salmonicida*). The results showed that almost 14% of the screened bacteria, originating from the cod larval environment, had antagonistic properties towards fish pathogens. The majority of these isolates were Gram-positive (81%). Only 3.2% of isolates could inhibit all three pathogens. *Aliivibrio salmonicida* was the easiest target, suppressed by 80.8% of the inhibitory isolates, while *V. anguillarum* was the least sensitive one, inhibited by 46.2% of isolates.

Based on partial 16S rRNA gene sequencing, inhibitory isolates were defined into three bacterial groups: Actinobacteria, γ -Proteobacteria and Firmicutes, the last one being the most abundant phylum of inhibitory isolates. The inhibitory Firmicutes isolates had the

broadest antagonistic spectrum and nearly all inhibited *Aeromonas salmonicida*. The Actinobacteria group A-C2 had the greatest inhibition towards *V. anguillarum* and isolate 04-342 (F-C3) towards *Aliivibrio salmonicida*. In contrast, Hjelm *et al.* (2004) found the inhibitory bacteria isolated from turbot larvae rearing units to be all Gram-negative, representing 8% of bacteria isolated and exhibiting *in vitro* anti-bacterial activity against three fish larval pathogens. Further, Fjellheim *et al.* (2007) reported the dominance of γ -Proteobacteria among antagonistic cod larval GIT isolates and based on several screening criteria determined *Vibrio*, *Pseudoalteromonas*, *Ruegeria* and *Microbacterium* spp. as probiont candidates (Fjellheim *et al.*, 2010). Caipang *et al.* (2010a) studied the antagonistic intestinal microbiota of wild cod selecting *Psychrobacter*, *Shewanella*, *Photobacterium* and *Vibrio* spp. as putative probionts. The importance of Gram-positive bacteria in our cod rearing environment was mainly linked to larval GIT and the algal concentrate, as well as dry feed and rearing water to some extent. This is an interesting finding since Gram-negative bacteria have been mainly linked to cod larval aquaculture (Brunvold *et al.*, 2007; Hansen & Olafsen, 1989). Some researchers have, however, reported the presence of LAB as an indigenous microbiota of cod (Seppola *et al.*, 2006; Strom & Olafsen, 1990). Others have demonstrated that *Pseudomonas* strains could be promising fish probionts (Gram *et al.*, 1999; Vijayan *et al.*, 2006). In fact, Spanggaard *et al.* (2001) reported the dominance of gills' pseudomonads while searching for bacteria antagonistic to *V. anguillarum* from the skin, gills and GIT of rainbow trout. A similar search was performed in our study, but the isolation of inhibitory bacteria from cod juvenile gills was not successful.

The fact that these Gram-positive bacteria and LAB showed great inhibitory activity towards Gram-negative pathogens is interesting since it is in contrast to the belief that the inhibitory spectrum of LAB is generally restricted to other Gram-positive bacteria (Abee *et al.*, 1995). Some LAB have, however, been reported to inhibit Gram-negative fish pathogens (Gildberg & Mikkelsen, 1998; Joborn *et al.*, 1997; Ringo, 2008; Robertson *et al.*, 2000). In the present study, the medium used for the well diffusion assay was found to influence the measured antagonistic effect, being greater when using MA than Tryptone Soy Agar (data not shown). Also, temperature used for antagonism testing can influence the results obtained (Caipang *et al.*, 2010a). This shows the limitation of this *in vitro* assay for probiont determination and exemplifies the need to select cultivation and incubation conditions closely resembling the environment where the probionts will be used. Due to differences observed in activity intensity and spectrum among the isolates tested, it is anticipated that the development of a mixture of probiotic strains would be more relevant to tackle the various

opportunistic or pathogenic bacteria that can arise in a rearing system at different stages. This was also pointed out by Fjellheim *et al.* (2010). Our results showed that most of the inhibitory isolates could be used in combination to others as little or no antagonism was observed among them. The mechanism of inhibitory action was not studied, but lowering of the medium pH, after bacterial cultivation at 15 °C, demonstrated that acid production could explain the inhibition caused by some isolates belonging to the Firmicutes phylum. Gatesoupe (1999) pointed out that the establishment of bacteria capable of producing lactic acid fermentation in fish digestive tract may inhibit the development of putrefactive microorganisms and therefore protect the host from microbial toxic metabolites. Also, it was found that all five Gram-negative inhibitory isolates were siderophore-producers, indicating their ability to survive and grow in iron-deficient conditions and nutrient poor environment. Such properties contributed to the control of pathogen growth in turbot larval rearing systems as shown by Gatesoupe (1997). Only one Gram-positive isolate (04-342) was found to produce extracellular inhibitory compound(s) under the conditions tested.

Temperature is an important parameter when it comes to bacterial growth. In the search of bacteria to be used to control the microbiota establishing in aquacultural systems, it is relevant to apply isolation techniques that ensure the retrieval of psychrotolerant or psychrotrophic bacteria as their optimal growth temperature may correspond to the conditions in cod rearing systems. The fastest growing inhibitory bacteria in the lower temperature range belonged to γ -Proteobacteria, while most Firmicutes isolates grew fastest in the upper temperature range. Such mesophilic bacteria are of relevance as a common problem at early larval stage is the heavy bacterial load of live feed enriched with fatty nutrients at 26 °C for several hours. The promising psychrotolerant bacteria (P-C8) were found to be haemolytic, a negative characteristic for a probiont. Therefore, the growth performance of other inhibitory isolates was considered within the temperature range anticipated in cod rearing and compared to pathogen growth. The maximum specific growth rate of a species is a fundamental character, both physiological and ecological. Lag time and doubling time have been proposed as the most important criteria for the comparison of bacterial growth curves (Vine *et al.*, 2004). Apart from P-C8 isolates, no other inhibitory isolates grew faster than *V. anguillarum* at 15 °C, but isolates CdV41, 04-394 and 04-683 outgrew *Aliivibrio salmonicida*. At 8 °C, i.e. close to the minimal cod rearing temperature, proliferation of pathogens was much delayed compared to two inhibitory isolates (04-279 and CdV41).

Probiont adherence to the host GIT is the first step in a probiotic treatment potentially leading to its transient colonisation, modulation of the immune system and antagonistic

activity against enteropathogens *in situ*. Adhesion capacity is therefore an important criterion for probiont selection. This characteristic was verified among six bacterial candidates and one pathogenic bacterium, *Aeromonas salmonicida*, in four different fish cell lines. Bacterial adhesion was influenced by the types of cell lines used, with EPC generally attracting fewer bacteria but CHSE most bacteria. This indicates the importance of evaluating bacterial adhesion capacity using more than one cell line type. LAB generally demonstrated good adhesion capacity. Adhesion of *V. anguillarum* was tested in EPC and observed to be poor in comparison to *Aeromonas salmonicida*, which adhesion capacity corresponded to that reported for *Aeromonas veronii* at 22 °C (EPC and RTG = $3.1 \pm 0.2\%$) by Rahman *et al.* (2002).

5.2 Selection of putative probionts

Five bacteria were retained for future investigations. Isolate 04-394 (*Enterococcus thailandicus*) has a broad and good inhibitory potential, with acceptable growth at 15 °C and good adhesion capacity. CdV41 (*Carnobacterium divergens* V41) had a broad inhibition spectrum, very good growth at 15 °C and a good adhesion capacity. Isolate 04-279 (*Arthrobacter bergerei*) had a restricted inhibitory spectrum, inhibiting only *V. anguillarum* but with great intensity, with acceptable growth at 15 °C and some adhesion capacity. A fourth isolate (04-683, *Enterococcus faecium*) had a similar temperature requirement as 04-394, a lesser inhibitory potential but a good adhesion capacity. The last one, strain 04-342 (*Alkalibacterium olivoapovliticus*) from the algal concentrate, was also selected because of its great antagonistic activity towards *Aliivibrio salmonicida* but its higher temperature requirement indicated its potential above 15 °C. Due to their low temperature tolerance, the other isolates could be applied in mixture as a rearing water supplement. Moreover, the addition of LAB or other probionts to control the bacterial development in live feed is a promising area. Rotifers and *Artemia* are filter-feeders and therefore appropriate vectors for probiont delivery to fish larvae. A commercial probiotic yeast (Levucell SB20[®]) was also evaluated in combination with two bacterial probionts during rotifer enrichment in 2005 (**Paper III**) because yeasts stimulate the fish immune system and may colonise the gut and accelerate its maturation at early stages (Gatesoupe, 2007).

5.3 Development of probiotic treatments (*Papers II, III, IV and V*)

During the development of probiotic treatments at early stages, one must consider the delivery mode, concentration and frequency of probiont application. This work is therefore based on (i) the few studies published prior to 2005 dealing with a bacterial treatment of ova or fish larvae to increase survival and (ii) the cod trial conducted in 2004 indicating how bacterial load is accumulating on ova postfertilisation until hatch. Ova treatment using bacteria added to the rearing water (10^{5-6} mL⁻¹, three days prior to and at hatching) was reported for halibut (Ottesen & Olafsen, 2000), resulting in an increased larval survival. Strom and Ringo (1993) evaluated larval bathing in a laboratory static system using *Lactobacillus plantarum* for 9 days with some water exchange, using a similar initial dose. This resulted in lower larval GI counts with a more homogeneous microbiota dominated by the added strain. Hjelm *et al.* (2004) assessed three doses (10^{3-5-7} mL⁻¹) of a *Roseobacter* strain applied to turbot larvae in a static system for six days and found the highest concentration to lead to increased survival. Similarly, Makridis *et al.* (2005) tested several bacteria (10^{5-6} mL⁻¹) added to sea bream larvae in a static system, leading to higher survival after 7 days for some strains.

These trials suggested that a probiont concentration of 10^{6-7} mL⁻¹ of rearing water may be appropriate. Further, a rapid bacterial growth was observed on disinfected cod ova incubated at 7-8 °C in our 2004 trial, reaching 10^6 g⁻¹ at midterm and maintaining this level until hatching (12-13 dpf). Therefore, this load appears to be a natural capacity for cod ova able to survive. Relating to the frequency of application, a short and repeated treatment was believed to be reasonable to build the “desired” ova microbiota, as it may be detrimental to stop the water flow for too long at this stage. Bathing close to hatch was avoided as ova tend to be fragile. Further, microbially overgrown ova may be less successful due to hypoxic conditions (Hansen & Olafsen, 1999). For larval treatment, a similar bathing treatment was designed, repeating the treatment for at least three weeks to frequently provide the selected probionts to larvae, starting before first-feeding and then slowly reducing the frequency in order to evaluate how long probionts will be maintained in larval gut post-treatment. Thus, three putative bacterial probionts (strains 04-279, 04-394 and CdV41) were tested in 2005 as a mixture added regularly to the rearing water from early postfertilisation to the larval mid-term stage in the experimental cod hatchery. This resulted in the determination of two prospective probionts (04-279 and 04-394) for bathing treatment (*Paper II*).

Two microbial treatments were applied to rotifer culture in order to assess another administration mode of probiotics via larval first-feeding. Live food organisms are an

appropriate vector for probiont delivery to fish larvae (Gatesoupe, 1994). In 2005, bacterial selection was based on inhibitory properties and growth potential of putative probionts at higher temperature for rotifer culture. Rotifers were enriched in the presence of the probiotic yeast, Levucell SB20[®], as well as bacterial strains 04-342 and 04-683 (**Paper III**). Treated rotifers were not successful as they led to high larval mortality, suspecting high *Vibrio* load to be the cause. Rinsing of enriched rotifers reduced their microbial load, but this was not performed for treated rotifers until 20 dph to avoid losing the microbial supplement. The loss of probiotic yeast during enrichment may suggest its utilisation by the growing rotifer microbiota, as demonstrated by a 100-fold *Vibrio* increase in treated rotifers compared to untreated ones. Indeed, Levucell was reported to drastically increase the bacterial load of undisinfected *Artemia* while reducing pollack larval growth. Conversely, its moderate use with another probiotic (Bactocell) in disinfected *Artemia* led to increased larval growth (Gatesoupe, 2002).

Methods to reduce microbial load of rotifers are not easily achieved in batch cultures, as demonstrated by our disinfection attempts that resulted in little microbial load reduction followed by the growth of tolerant bacteria during enrichment. Similarly, Hoj *et al.* (2009) reported that despite the reduced abundance of culturable heterotrophic bacteria associated with enriched nauplii induced by a cleanup step including formalin, Virkon[®] S and DHA Protein Selco, a concurrent relative enrichment of *Vibrio* DNA in the overall bacterial DNA pool was observed. This demonstrates that *Vibrio* cells were relatively more resistant to the antimicrobial treatment applied, though it was not shown whether the detected DNA originated from active cells capable to grow. It was therefore suggested that disinfection of *Artemia* could only be meaningful if used in combination with probiotics to ensure beneficial bacterial dominance post-treatment.

In 2006, another commercial probiotic product, Remus[®], was supplemented to enriched rotifers 30 min prior to larval feeding. This probiotic treatment was compared to untreated larvae and those bathed with the *Enterococcus* and *Arthrobacter* probionts (**Paper IV**). Makridis *et al.* (2000) showed that bacteria can be bioencapsulated within 20–40 min in rotifers. In our study, the Remus-treatment led to significantly lower larval survival and lesser growth along with significantly higher levels of haemolytic vibrios in larval GIT when compared to control and bathed larvae. The microbial load of rotifers was apparently not influenced by the Remus-treatment, but haemolytic vibrios accounted for 10% of TCBS count on 14 dph while being undetected in regular rotifers. Mass cultivated live feed has been shown to contain bacteria detrimental to larvae (Benavente & Gatesoupe, 1988) and a high

mortality has been linked to proliferation of haemolytic bacteria in turbot (*Scophthalmus maximus* L.) larvae after live feed introduction (Salvesen *et al.*, 1999). In a recent study, a combined Remus[®] treatment via the rearing water and rotifer inoculation at early cod stage led to better larval survival but no growth enhancement was observed (Sveinsdottir *et al.*, 2009).

Finally, probiont-coated dry feed was prepared to evaluate the single and mixed application of the two prospective probionts at early juvenile stage for a short (28 days) or extended (55 days) period (**Paper V**). Use of probiotic dry feed has been reported for several juvenile species, with adequate doses ranging between 10^{6-9} CFU g⁻¹ feed (Bagheri *et al.*, 2008; Ghosh *et al.*, 2008; Panigrahi *et al.*, 2004; Vendrell *et al.*, 2008). Frequent application has been suggested to contribute to probiont maintenance in the host digestive tract to achieve the desired effects (Gatesoupe *et al.*, 1999; Macey & Coyne, 2006; Nikoskelainen *et al.*, 2003; Panigrahi *et al.*, 2004). Further, Taoka *et al.* (2006) and Brunt and Austin (2005) showed the greater efficacy of viable cells incorporated to the diet compared to dead cells or cell-free supernatant.

Probiont viability should therefore be verified in probiotic feed. Irianto and Austin (2002) reported a declining activity of probionts incorporated into diets over a two-month period. Our preliminary study showed that both bacteria were suitable to produce probiotic feed with an adequate dose of viable cells lasting for at least 8 weeks when stored between -20 °C and 15 °C. In more, feed quality evaluation revealed that bacteria-coating of feed caused little or meaningless change, while frozen storage initiated the oxidative process in both control and probiotic feeds based on faint oxidative off-odours detected by the sensory panel (**Paper V**). This is an important finding since increased peroxidation stress may account for poor fish growth and survival, as demonstrated by Tocher *et al.* (2003) who induced a stressful pro-oxidant status by feeding oxidised oils to weaned marine juveniles. This emphasises the importance of proper storage conditions for larviculture feeds and nutrients.

5.4 Revealing two prospective probionts: their safety and potential

The application of three putative bacterial probionts (strains 04-279, 04-394 and CdV41) in the cod hatchery as a mixture added regularly to the rearing water from early postfertilisation revealed two prospective candidates (**Paper II**). Strain CdV41 was not detected in bathed larvae and its use was therefore discontinued in subsequent trials. Strains 04-279

(*Arthrobacter bergerei*) and 04-394 (*Enterococcus thailandicus*) were re-isolated in larval GIT at the end of the experiment, i.e. 7 days post-treatment, contributing to increased survival, growth, viability and microbiota control (**Paper II**), thus fulfilling several criteria for probiotic candidates (Gatesoupe, 1999; Verschuere *et al.*, 2000). Undertaking a similar bathing trial with the two prospective probionts from the late prehatch stage in 2006 led to significantly enhanced larval growth and development, and influenced the GI microbiota (**Paper IV**), confirming most of earlier findings. Importantly, the non-pathogenicity of both probionts was evidenced following i.p. injection of cod juveniles as neither pathological effect nor death was observed (**Paper IV**). In more, the enzymatic potential of both probionts was demonstrated, suggesting their possible contribution to larval digestion and uptake of nutrients as well as increased growth. It has also been reported that probiotics can increase digestibility (Lin *et al.*, 2004) and enzymatic activity (Macey & Coyne, 2006) in aquatic animals.

The *Enterococcus* genus, which strain 04-394 belongs to, has been linked to pathogen antagonism, a main feature of candidate probiotics (Gatesoupe, 2008), and may explain the microbiota control towards *Vibrio* spp. observed *in vivo* (**Papers II, III and V**). In fact, antagonism of strain 04-394 towards *Vibrio* spp. was seen *in vitro* as well (**Paper I**). Our data points out a strong relation between strain 04-394 with larval survival and growth and lower *Vibrio* levels in larval gut as evaluated by PCA analysis in the 2005 trial (**Paper II**). Several studies have reported the probiotic effects of *Enterococcus* strains on fish. Bogut *et al.* (2000) have shown the positive effects of *Ent. faecium* administered to sheat fish on its intestinal microbiota, reducing harmful bacterial load, and its increased weight gain. Feeding of *Ent. faecium* in eel through a 14-day diet influenced the gut microbiota and increased disease resistance (Chang & Liu, 2002). Recently, Swain *et al.* (2009) reported the antagonistic effects of *Ent. faecium* in shrimp, reducing vibriosis in challenge trials. Furthermore, Wang *et al.* (2008) suggested the increased growth performance of tilapia treated regularly with *Ent. faecium* to be attributed to the improved immune responses. Panigrahi *et al.* (2007) have reported on the immune system modulation of rainbow trout fed a *Ent. faecium* diet.

Despite the better adhesion properties of strains 04-394 and CdV41 on different fish cell lines (**Paper I**), strain 04-279 (*Arthrobacter* sp.) dominated in bathed larvae in 2005, which may be explained by its 10-fold higher concentration during bathing as compared to the other strains added (**Paper II**). Bacteria of the genus *Arthrobacter*, originating from various niches, may have proteolytic and antagonistic properties (Hentschel *et al.*, 2001; Jayanth *et al.*, 2001; Smacchi *et al.*, 1999), and produce valuable substances, such as amino acids,

vitamins, enzymes, specific growth factors, pigments and polysaccharides (Abrashev *et al.*, 1998). An interesting property of some *Arthrobacter* spp. include the production of AHL lactonase, enabling quorum quenching and hence interfering with neighbouring bacterial communication. The enzyme role is still unclear but it is likely to allow for successful competition and nutrient acquisition in a complex microbiota (Czajkowski & Jafra, 2009). This ability was however not verified during the study with the corresponding strain 04-279. Another property of strain 04-279 was demonstrated by the API ZYM test, i.e. the active production of α -mannosidase, an enzyme involved in the cleavage of α -mannose (*Paper IV*). Transformation of mannose to mannuronic acid by bacteria has been proposed (Remminghorst & Rehm, 2006). Mannuronic acid is a non-specific fish immunostimulant (Skjermo *et al.*, 2002). Bacteria belonging to the genus *Arthrobacter* have been isolated from the digestive tract of fish (Ringo, 1993; Ringo *et al.*, 2006). Recently, an *Arthrobacter* strain was reported to improve the water quality and survival in shrimp culture by degradation of dissolved nitrogen (Li *et al.*, 2006). Another strain, licensed as a salmonid vaccine against selected pathogens, promoted growth and health of haddock larvae by influencing the larval microbiota (Plante *et al.*, 2007).

5.5 Rearing variability and impact of prospective probionts on cod survival

The impact of the two prospective probionts on survival of treated larvae and juveniles was found to differ among trials. It has been reported that larval variability, their health and ontogeny as well as stochastic factors and conditions developing in rearing systems may lead to differences in the microflora established (Carnevali *et al.*, 2004; Fjellheim *et al.*, 2007; Ringo & Birkbeck, 1999; Tinh *et al.*, 2008a). This probably explains the highly variable survival observed in apparently controlled hatchery rearing (Bjornsdottir *et al.*, 2009), as seen in our control and treated groups (*Paper II*). The variability of survival rate within bathed and control groups in 2006 (*Paper IV*) was reduced compared to the previous study in 2005 (*Paper II*). This could be due to better quality ova collected during early spawning (Marteinsdottir & Steinarsson, 1998), improved disinfection method and feeding regime used in the 2006 study (*Paper IV*). The lower average survival rate of bathed larvae in 2006 compared to the untreated group could be due to the intermitted water flow accident which occurred at 4 dph, lasting for 12 h in all bathed silos, and may have been detrimental to the young larvae causing losses that could not be estimated at that early stage. Survival data from the 2006 trial should therefore be interpreted with caution. Average cod larval survival rates at

the hatchery involved in the study ranged between 10-12% in 2003-2007, with a maximal rate being 18-28% (A. Steinarsson, oral communication).

5.6 Impact of prospective probionts on cod growth, viability and development

Enhancing larval growth, development and viability are valuable characteristics for probiotics. The probiotic bathing treatment led to increased larval dry weight, observing a 19.3% increase in 27-dph larvae in 2005 (*Paper II*) and 23.5% in 36-dph larvae in 2006 as well as a 5.6% increase in length (*Paper IV*), compared to untreated larvae. Difference in weight gain was seen among treatments despite the same live feed quantity provided daily. This may indicate the contribution of the established microbiota in larval digestion, since larvae have a poorly developed digestive system at early life (Olafsen, 2001). Cod larval length (8-14 mm) has been positively related to the length and volume increase of the midgut epithelium, with increased alkaline phosphatase activity in the brush-border membrane (Wold *et al.*, 2007). Thereby, the 100-fold higher total culturable microbial load observed in GIT of bathed larvae in 2006 (*Paper IV*), compared to untreated ones, may have resulted from an increased midgut epithelium volume and played a role in better feed utilisation, influencing larval development (Suzer *et al.*, 2008). Promoting larval growth is certainly an important feature since recent findings show that growth difference during first-feeding of cod persisted after 30 months in sea-pens (Imsland *et al.*, 2006).

Bacterial bathing significantly enhanced larval viability as demonstrated by the hypersalinity tolerance test performed in the 2005 trial (*Paper II*). In comparison, Rollo *et al.* (2006) used a pH stress test to demonstrate the significant improvement in tolerance of sea bream (47 dph) fed LAB-treated live feed. In intensive rearing systems, stress is generated in many ways but needs to be reduced to a minimum to optimise the chances for survival. It has been proposed that since protein turnover is energy costly, there may be a trade-off between fast growth and stress-resistance/survival in fish larvae (Conceicao *et al.*, 2001).

Further, the juvenile intervention study demonstrated that probiont administration via feeding contributed to enhanced cod growth and survival under the non-optimal rearing conditions tested (*Paper V*). The results clearly indicated the superior effects of the *Enterococcus* strain compared to *Arthrobacter* strain. In this study, transfer of juveniles to the experimental tanks with a short acclimatisation period was apparently stressful, resulting in a decreased appetite and low growth rates for the first period. This stress may be attributed to

transfer to lower temperature. Optimal growth temperature for 10-g cod juveniles is 13.6 °C based on the model developed by Bjornsson *et al.* (2007), while the optimal FCR temperature for 6-17 g juveniles is 12 °C (Bjornsson *et al.*, 2001). At 8 °C, FCR was found to be 1.4-1.7 in a 41-day trial of 12-g juveniles with a growth rate of 2% day⁻¹ (Bjornsson *et al.*, 2001). This expected SGR was not reached in the latter part of our experiment despite the considerable increase seen for the groups tested, indicating the non-optimal rearing conditions of our study. However, the lower FCR of the 55-day period than that of the first 4 weeks demonstrates that feed was better utilised or eaten by the juveniles during the second month, explaining the higher SGR observed. Olafsen (2001) has suggested that stress reduced the resistance against infection, and it favoured proliferation of opportunistic bacteria or unpredictable development of bacterial communities.

In the 2006 trial, larval development was assessed by proteolytic activity of larval lysates and immunological analysis of important proteins for cod at early stage (**Paper IV**). The increased proteolytic activity detected 4-14 dph in bathed larvae compared to those untreated further supports that an advanced development of the digestive tract of bathed larvae occurred. This may have contributed to the absorption of nutrients since the same live feed ration was provided to all silos. In addition, significant growth increase of bathed larvae may be attributed to the enzymatic potential of the probionts, contributing to larval uptake of essential amino acids (leucine and valine) and digestion of more complex molecules, hence stimulating larval digestion. Cod larvae lack a stomach at first-feeding and therefore absorb free amino acids more efficiently than those in polymerised form (Rust *et al.*, 1993). Leucine, liberated by both probionts, is the precursor of several compounds involved in important physiological functions (Aragao *et al.*, 2004).

Faster larval development was also demonstrated 4 dph by the proteins present in bathed larvae after only three bathing treatments, while not or faintly detected in the untreated groups (**Paper IV**). The most prominent protein corresponded to apoLP A-I (30 kDa) and/or a 28 kDa protein previously described at onset of cod feeding (Magnadottir *et al.*, 2004). Haemoglobin was detected at 4 dph in bathed larvae or earlier than previously reported (10 dph) by Magnadottir *et al.* (2004). In contrast to the findings of Magnadottir *et al.* (2004), C3 was not detected in our study, probably due to the lower sensitivity of the detection method used. The enhanced presence of apoLP A-I in bathed larvae at early posthatch may reflect the positive influence of the probionts on larval development and immune modulation. ApoLP A-I is hydrophobically associated with C3 in cod plasma and has a regulatory role in the complement system (Magnadottir & Lange, 2004). Recent findings have suggested that it

plays a role in apoptosis and is involved in organogenesis and homeostasis of various cod organs (Lange *et al.*, 2005).

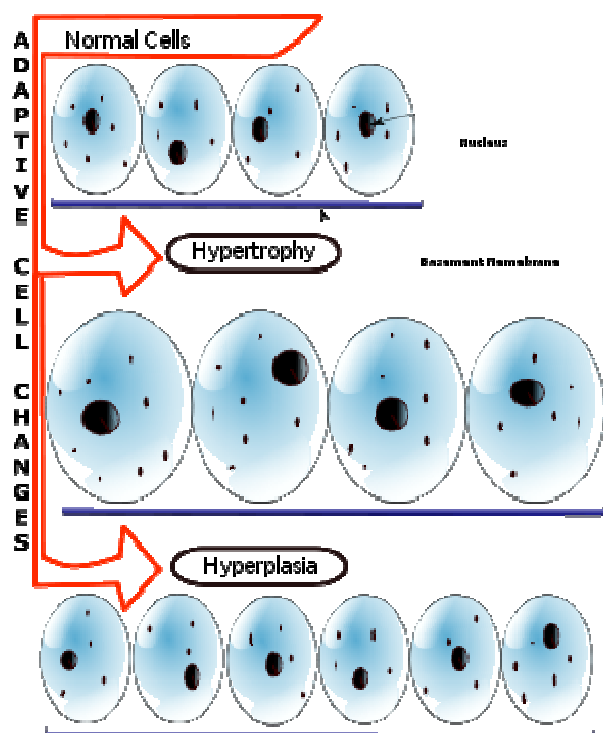
5.7 Maintenance of probionts added to the rearing system

Maintenance or establishment of probionts in rearing systems is a pre-requisite for a proof of effects. Presence of added bacteria was evidenced in the rearing water up to three days following bathing at the prehatch stage but generally not after 5 or 7 days posthatch. This information should be considered in the design of a bathing scheme. LAB had established on bathed ova prior to hatching and their microbial load reflected that observed on resulting larvae 2 dph, indicating a bacterial transfer from ova to larvae as proposed by Hansen and Olafsen (1989). In fact, 16S rRNA gene sequencing confirmed the presence of the added strain 04-394, 2 dph on larvae originating from bacteria-treated ova. Further, the presence of two of the added bacteria in larvae from the most successful silo was similarly confirmed 27 dph in 2005, i.e. 7 days after the last bathing treatment. The probionts represented together 30% of the culturable gut microbiota (**Paper II**). A lower probiont concentration (0.001-0.3% of TVC for 04-394 and 0.004-0.07% of TVC for 04-279) was observed 8 days post-treatment in a similar trial in 2006 (**Paper IV**). However, these bathed larvae were older (36 dph), approaching metamorphosis and with a 100-fold higher gut microbial load than seen in control larvae or all other larval groups in 2005. The impact of these parameters on the larval microbiota composition was not evaluated in the trial performed in 2006.

The level difference observed between re-isolated probionts and the total culturable GI microbiota of cod larvae in 2006 does not necessarily indicate that probionts were a minority group among the autochthonous gut microbiota. In fact, the whole GI microbiota was analysed, but not that solely associated with the gut wall. Resident and transient gut microbiota in older fish has been reported to differ, with a lesser bacterial abundance (10- to 10000-fold) and diversity in resident microbiota (Merrifield *et al.*, 2009; Ng *et al.*, 2009; Ringo *et al.*, 2006; Spanggaard *et al.*, 2000). Whether a transient gut microbiota gains more importance over the resident one as a cod larva and its GIT develop is not known. This is though a possible explanation taking into account that both cell hyperplasia and hypertrophy contribute to the increase in midgut size at early larval stage. But when larval length surpasses 10-11 mm, hypertrophy alone has been shown to occur (Wold *et al.*, 2008). This implies that larger cells may offer less bacterial attachment space than smaller and more numerous cells may do, as shown in Figure 16. This developmental change may result in apparently fewer

resident bacteria in the gut while the increased midgut size may at this stage support higher levels of transient bacteria.

Colonisation of added bacteria in bathed marine fish larvae at early stage (3-6 dph) has been demonstrated (Makridis *et al.*, 2000; Makridis *et al.*, 2008; Plante *et al.*, 2007; Ringo, 1999) and emphasises the importance of an early treatment to influence the larval microbiota.



Ringo and Vadstein (1998) demonstrated the importance of an early probiotic supplementation to the rearing water before live feed introduction to effectively colonise the larval gut as the first possible bacterial accumulation occurs via drinking. Delaying it after first feeding can lead to a lower colonisation level of added bacteria.

Figure 16. Illustration showing the difference between cell hypertrophy and hyperplasia (retrieved 17.09.2010; <http://en.wikipedia.org/wiki/Hyperplasia>)

During the intervention study, cod juveniles were fed daily with probiont-coated dry feed (*Paper V*). It can be foreseen that probiont leakage from the feed to the rearing water may have happened to some extent. Based on the average daily feed ration, maximum probiont levels in the rearing water could have reached 3.5 and 4.8 log CFU ml⁻¹ for *Enterococcus* and *Arthrobacter* probionts, respectively. Probionts were presumptively isolated from juvenile gills at 10- to 100-fold lesser levels, indicating some probiont leakage to the rearing water from the feed or even the excrements. The probionts were though not detected in the rearing water 12-h post-feeding, probably due to the water exchange and the low level lost from the feed. Further, probiont survival throughout the digestive tract was not evidenced since digesta or faeces analysis was not performed. Nevertheless, the *Enterococcus* probiont was presumptively detected from the gut of juveniles administered the mixed probiotic feed for 28 days, while probionts' level in the gut was lower than 200 CFU g⁻¹ after 55-day feeding of single-probiont feed. It is though likely that the probionts administered daily were part of the transient microbiota.

5.8 Effects of hatchery treatments and prospective probionts on microbiota control and composition

Culturable microbial load control by the prospective probionts was generally limited. Regular bacterial bathing of ova led to lower bacterial counts compared to untreated rearing systems prior to live prey feeding (**Paper II**), indicating some microbiota control as reported following bathing of cod larvae with *Lactobacillus plantarum* (Strom & Ringo, 1993). As the trials progressed, little difference was observed in TVC or presumptive *Vibrio* counts of larvae or rearing water. However, the intervention study in cod at early juvenile stage demonstrated that administration of the two prospective probionts, either mixed (28-day trial) or singly (55-day trial) via dry feed (10^{7-9} CFU g⁻¹), modified the gut microbiota (**Paper V**). Combined probiont application during 28 days resulted in *Vibrio* control in cod juvenile gut, while lowering the total gut microbiota load significantly compared to the control group. *Vibrio* levels were maintained in the gut of juveniles fed probiotics separately for 55 days, though only significantly in presence of the *Enterococcus* strain. Moreover, use of single probiont feed led to a better microbial control of the rearing water after 55 days. This is in accordance with other studies, as probiotic feeding trials have reported decreased gut microbiota load, e.g. in eel (Chang & Liu, 2002), tilapia (Jatoba *et al.*, 2008) and ornamental fish (Ghosh *et al.*, 2008).

Quantitative microbiological evaluation of rearing systems alone has proven to be less informative than when performed in conjunction with their qualitative analysis (Bjornsdottir *et al.*, 2009; Makridis *et al.*, 2000; Ringo *et al.*, 1996; Ziaei-Nejad *et al.*, 2006). This is in agreement with our findings where TVC of larval GIT were similar for the differently treated silos at the end of the experiment despite different larval development or performance (survival and stress tolerance). The results showed that microbiota composition affected larval survival and growth rate (**Papers II and III**). Further, culturable microbiota analysis revealed the inaccuracy of *Vibrio* counts obtained from TCBS medium, being occasionally overestimated or underestimated, as indicated by the growth of non-vibrio genera on the medium or by some undetected *Vibrio* species otherwise present on marine agar, respectively (**Paper III**). Lopez-Torres and Lizarraga-Partida (2001), Hoj *et al.* (2009) as well as Prol-Garcia *et al.* (2010) recognised a similar TCBS selectivity deficiency when analysing rotifer and *Artemia* samples. Further, Reid *et al.* (2009) reported a *Vibrio* phenon, genotypically identified as *V. pacinii*, that did not grow on TCBS medium following replica plating from MA when analysing cod larvae fed rotifers. However, *V. pacinii* has been reported to grow on

TCBS (Gomez-Gil *et al.*, 2003). It is therefore likely that such vibrios can grow on TCBS medium when streaked separately, as in our case, but growth inhibition by nearby antagonistic colonies on the plate may have occurred.

Moreover, our studies demonstrated that haemolytic vibrios were present prior to hatching and prevalent at several stages of cod rearing (**Papers II, III and IV**), therefore not explicitly linked to live feed. They were found in enriched *Artemia*, rearing water and larvae from large scale rearing systems in 2004; on control ova and larvae, both newly hatched and 27 dph, as well as in rearing water and enriched *Artemia* in 2005; on ova 10 dpf, in the rearing water at pre- and posthatch stages as well as in larvae in 2006. At the juvenile stage (**Paper V**), haemolytic bacteria were also detected in the rearing water and on the gills. Enumeration of presumptive vibrios in 2006 (**Paper IV**) showed comparable levels between bathed and control while replica plating to blood agar indicated a 50-fold lower incidence of haemolytic vibrios in bathed larvae ($P>0.05$). Based on the possible negative effect of such bacteria on larval and juvenile health, it is imperative to understand their role in rearing systems and determine means to control their growth. Schulze *et al.* (2006) studied the bacterial diversity in marine rearing systems and found several bacterial species to be haemolytic, nearly all of them being gamma-Proteobacteria and many isolated from moribund larvae. Recently, Alexeeva *et al.* (2004) investigated the impact of cultivation conditions on haemolytic activity of *Pseudoalteromonas issachenkonii* and found that growth and haemolytic activities were greater on sea-salt-based media rather than on NaCl-containing media. This finding may indicate that our study might underestimate the importance of haemolytic bacteria in cod rearing since a sheep blood agar containing 2% NaCl was used. Further, it was observed in the juvenile trial that plating on such blood agar in parallel to MA resulted in lower counts, indicating that not all culturable microbiota could growth on the blood agar.

The dominant culturable microbiota of differently treated cod rearing systems was studied postfertilisation and posthatch during the 2004 and 2005 hatching periods (**Paper III**). Cultivation methods were used to retrieve bacterial isolates and allow for their enumeration and characterization, which could not be achieved by culture-independent methods. Further, DNA fingerprinting methods for bacterial community screening may be biased due to DNA extraction as well as the problem of heterogeneity between copies of 16S rDNA on the bacterial genome leading to multiple bands from one bacterium, as discussed in Brunvold *et al.* (2007). Although cultivation methods may introduce some bias, especially due to the presence of non-culturable bacteria, this problem may be of less importance in a nutrient-rich hatchery environment, assuming use of proper media and incubation methods. A phenotypic

and genotypic analysis of dominating bacteria was conducted along with the assessment of their metabolite production, such as haemolysin, siderophores and AHLs. This contributed to a better understanding of the effects of treatments on the microbiota composition, relating rearing performance to the microbiota characteristics. Overall, our results demonstrate that a diversified microbiota prevailed in cod rearing systems, influenced by both the treatments and feeds provided. Phenotypic tests demonstrated the heterogeneity within some OTUs defined by partial 16S rRNA gene sequencing. Multivariate analysis of larval microbiota and corresponding survival data in larval silos in 2005 divided the genotypic groups into beneficial/harmless and detrimental/opportunistic clusters. Putative virulence factors (siderophores and AHLs, mainly) were common among the proposed detrimental/opportunistic OTUs.

Genotypic analysis of phenotypically representative bacterial isolates depicted the great bacterial diversity encountered in the cod rearing environment with 75 OTUs. Similar trends were observed in the hatchery rearing systems during the two periods studied, with prevailing γ -Proteobacteria prior to hatching towards a multiphyla microbiota posthatch. A homogeneous microbiota of wild, newly fertilised ova was defined, but it differed slightly between the two periods investigated. *Marinomonas* sp. dominated in 2004 in contrast to *Colwellia* sp. and an unknown γ -Proteobacterium in 2005. This indicates that environmental factors also influence microbiota composition and activity, thereby possibly altering ova development. Glutaraldehyde disinfection led to the survival of tolerant bacteria, like *Pseudoalteromonas* sp. in 2004, but a more diversified microbiota resulted in 2005 suggesting handling contamination in the hatchery. The dominating OTUs apparently indigenous to newly fertilised ova had re-established prior to hatching despite their disappearance following disinfection. *Marinomonas*, *Colwellia* and *Pseudoalteromonas* spp. are common rearing water bacteria (Jensen *et al.*, 2004), which have been found on cod ova or unfed cod and halibut larvae (Bjornsdottir *et al.*, 2009; McIntosh *et al.*, 2008), and may therefore have a certain ability to adhere to ova. Healthy ova hatch sterile larvae which rapidly become contaminated by ova debris and rearing water microbiota (Hansen & Olafsen, 1989). Such a colonisation trend was observed in 2005, with prevailing *Pseudoalteromonas* and *Colwellia* spp. on newly hatched larvae.

After few weeks of exogenous feeding, the link between feeding diets and larval microbiota was evidenced, as reported by others (Blanch *et al.*, 1997; Grisez *et al.*, 1997; Korsnes *et al.*, 2006; Reid *et al.*, 2009). Generally, the presence of three rotifer bacterial OTUs (*Lacinutrix*, *Microbacterium* and *Pseudoalteromonas* spp.) in larval GIT accounted for

40-70% of the microbiota, not considering the 10-20% *J. marina*/*Flavobacterium* level found in some larval groups and possibly originating from rotifers based on microbiota analysis in 2004. Bacterial isolates of two novel Flavobacteriaceae genera were identified in rotifers and larvae, *Lacinutrix* sp. (Bowman & Nichols, 2005) and *J. marina* (Quan *et al.*, 2008). Similarly, 28- and 56-dph larvae in 2004 harboured several of the bacterial groups found in enriched *Artemia* and dry feed. Several genera isolated from larvae in our study have been reported in recent cod microbiota analysis, using either cultivation (Korsnes *et al.*, 2006; Reid *et al.*, 2009) or culture-independent (McIntosh *et al.*, 2008) methods. Antibiotic treatment applied 55 dph as remedy to the problematic fish health situation in tank 1 reduced the microbiota diversity considerably and resulted in two dominant OTUs, *Psychroserpens burtonensis* and *Pseudomonas stutzeri* constituting 90% of the culturable microbiota (56 dph).

5.9 Beneficial and detrimental bacteria in cod hatchery rearing (*Paper III*)

Comparison of the differently treated larval silos in 2005 indicated that *Vibrio* spp. were not the only undesirable bacteria found in larval GIT since their low levels did not always result in better survival. Obviously, other bacterial groups were detrimental to cod larvae. Analysis of the contribution of the different larval genotypic groups to larval survival discerned two main clusters, beneficial/harmless and detrimental/opportunistic bacteria. The former group includes the *Enterococcus* and *Arthrobacter* probionts, two *Pseudoalteromonas* phenotypes and one *Lacinutrix* phenotype, all isolated from bathed larvae (S1), while six genotypic groups are suggested to negatively affect larval health. Putative virulence factors (PVFs), i.e. haemolytic, siderophore and AHL properties, detected among four of them strengthen this view. *V. splendidus*, a common planktonic bacterium (Nealson *et al.*, 1993) isolated from poorly surviving larvae and expressing the three PVFs, was already present at postfertilisation stage while not being found in live feed, suggesting its introduction to the hatchery via seawater. A selective advantage of *V. splendidus* over other live feed vibrios, like *V. alginolyticus*, has been reported in turbot larval gut (Thomson *et al.*, 2005) and could be due to its ability to thrive at lower temperature (<20 °C) (Maeda *et al.*, 2003). Further, the pathogenicity of *V. splendidus* was recently reported in cod larvae (Reid *et al.*, 2009). Bruhn *et al.* (2005) also detected AHL from *V. splendidus* isolates virulent to turbot larvae (Thomson *et al.*, 2005). Further, we suspect GP-C4 *Vibrio* isolates not detected on TCBS medium (99% similar to *V. gallicus*, *V. hepatarius*, *V. xuii*) to be detrimental, producing siderophores and AHLs. They originated from treated rotifers and larvae with low survival,

i.e. those fed on these rotifers (S5+6) or from the adjacent silo (S4) where silo cross-contamination may have occurred. Austin *et al.* (2005) studied the pathogenicity of these *Vibrio* species to rainbow trout (*Oncorhynchus mykiss*, Walbaum) fry and *Artemia* nauplii, demonstrating either non- or low virulence in these animal models. *V. xuii* has been associated with rotifers and moribund cod larvae (Brunvold *et al.*, 2007).

Pseudoalteromonas isolates studied showed heterogeneous phenotypic traits, as reported by Ivanova *et al.* (2004), which could explain a variable nature, either beneficial, harmless or detrimental. Siderophore production was detected among most of them, but important producers were found among a minority (25%) of isolates, while none caused haemolysis and only 7.7% of those tested produced AHLs. Some are known to produce antimicrobial substances (Isnansetyo *et al.*, 2009; Longeon *et al.*, 2004), metalloproteases (Lee *et al.*, 2002; Miyamoto *et al.*, 2002) and may be pathogenic (Pujalte *et al.*, 2007). *Psychrobacter celer*, a siderophore producer, was only isolated from poorly surviving larvae (S5+6). This species was recently identified from the Korean South Sea environment (Yoon *et al.*, 2005), with its name denoting its rapid growth properties. Lastly, it is not clear whether *J. marina*/*Flavobacterium* sp. and *Microbacterium oxydans* were autochthonous microbiota members or harmful to larvae since PVFs were not detected *in vitro*. Some *Flavobacterium* sp. are pathogenic to fish, mainly salmonids, and have been detected on cod eggs but not in association with disease (Hansen & Olafsen, 1989). It is noteworthy that *J. marina* has a wide enzymatic potential (H.L. Lauzon, unpublished data) and might have a role in larval GIT. Ringo *et al.* (2006) recently isolated *Microbacterium oxydans* adhering to the hindgut of farmed Atlantic salmon. Orozco-Medina *et al.* (2002) reported *Microbacterium* sp. on *Artemia* cysts with positive or negative effects on growing *Artemia* depending on the isolates. Prol-Garcia *et al.* (2010) distinguished a *Microbacterium* sp. from the bacterial community of rotifers, while Reid *et al.* (2009) isolated this genus from cod larvae fed rotifers.

We observed two different bacterial consortia leading to lowest larval survival (5%) with one common *Vibrio* phenotype, a siderophore- and AHL-producer inducing both monitor bacteria. Both consortia counted another AHL-producer, *V. splendidus* or *Lacinutrix* sp. found at high levels, where interspecies communication may have occurred. Communication through QS enables bacterial populations to coordinate their behavior. Our study used only two AHL-monitor bacteria and may therefore underestimate the ability of the cod microbiota to produce different QS signal molecules. CV026 responds mainly to short-chained unsubstituted AHLs (McClean *et al.*, 1997), while pZLR4 detects a broad range of AHLs: oxo-, hydroxy- and unsubstituted-AHLs (Cha *et al.*, 1998). In this study, 95% of positive

AHL isolates induced pZLR4. Based on other biosensors, Tait *et al.* (2009) revealed several other genera involved in QS, namely *Shewanella*, *Marinobacterium*, *Glaciecola*, *Sulfitobacter* and *Rhodobacter* spp., with some of them also involved in QQ due to their ability to degrade AHLs.

6 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The research work presented was initiated in 2004 and aimed to fulfill several gaps in the knowledge relating to cod aquaculture microbiology at early developmental stages where a high larval mortality rate is the main bottleneck of juvenile production. Specific objectives were to be met in order to achieve better survival and to promote cod development at early rearing stages, targeting the most critical period. This was attempted by developing probiotic methods to control the microbiota and to improve cod performance. Several research questions were to be answered.

Firstly, whether the natural microbiota of cod rearing environment did harbour potential probiotic bacteria and whether the use of pre-defined *in vitro* tests could lead to the selection of putative probionts. A rather conservative and wide approach was instituted to ensure a good research foundation. The search for tailor-made probiotics to suit the early stages of cod rearing included the use of diverse microbiological media, a wide range of samples relating to the first few months of cod life and several *in vitro* tests to characterise the putative probionts and to select few candidates based on criteria assumed to suit cod rearing environment. This screening for probiotics among cod rearing microbiota using multiple tests led to our publication in 2008 (*Paper I*).

In the next step, the probiotic application method, dose and frequency were to be experimented and developed based on available literature and current cod rearing information. The development of probiotic methods involved testing the few candidates *in vivo* at pre- and posthatch stages in a cod hatchery. This methodology has some drawbacks since it is time-consuming and costly. Hence, it reduced our possibilities to evaluate the effect of candidate concentration and combination on larval success. An *in vivo* multiwell method for candidate testing as performed by Fjellheim *et al.* (2010) would have been a practical, additional screening step in our study prior to large scale testing in the hatchery. However, the short spawning period and little availability of cod ova limited our research work considerably. Nevertheless, our approach led to the determination of two prospective probionts, the first

reported for bacterial bathing treatment at early cod stages leading to increased larval survival, growth, viability and microbiota control (*Paper II*).

Two trials in the successive year allowed the validation of the probionts at larval (*Paper IV*) and juvenile (*Paper V*) stages using different administration modes; rearing water and probiont-coated dry feed, respectively. It was therefore demonstrated that the probionts could be used at both stages and by both vectors. The larval trial confirmed most of earlier findings, but did not assess the potential of each probiont separately. This was verified in the juvenile trials. The larval trial also revealed the contribution of the probionts in faster larval development and in the stimulation of the larval GI microbiota development while maintaining haemolytic vibrios at low levels (*Paper IV*). The juvenile trials clearly showed that both probionts, especially the *Enterococcus* strain, modified the gut microbiota and contributed to enhanced juvenile growth and survival. In fact, the results indicated that the single use of the *Enterococcus* probiont during juvenile feeding was the best alternative (*Paper V*). Further, the non-pathogenicity of the probionts was confirmed in larvae and juveniles (*Paper IV*).

Meanwhile, development of probiotic rotifers was attempted using different probiotic candidates but failed (*Papers III and IV*). The main obstacle was the high microbial contamination maintained by the batch culture conditions. Further, disinfection attempts led to the tolerance and growth of undesirable microorganisms during enrichment. Alternative methods, like the development of gnotobiotic rotifers by cyst treatment and culture in a recirculating system matured by desirable bacteria, may remedy to this problematic situation. This was, however, not attempted in the study due to the lack of a recirculating system. Treating rotifer cultures with the two promising probionts was not evaluated, but preliminary *in vitro* testing of the probionts' antagonistic properties towards some rotifer bacterial isolates indicated their potential in controlling the rotifer microbiota (H.L. Lauzon, unpublished data).

The study also demonstrated that the changing environmental parameters in cod rearing are reflected in the developing microbiota of rearing water, ova and larvae where live feed introduction and treatments as well as bacterial associations play a crucial role in rearing success (*Paper III*). In fact, the specific objectives sought to compare the culturable microbiota of treated and untreated ova and larvae and to define positive and negative parameters influencing cod survival and development. It was found that differences in larval survival coincided with the diversified larval GI microbiota observed in the differently treated silos, discerning two main clusters, i.e. beneficial/harmless and detrimental/opportunistic bacteria. The former group included both *Enterococcus* and *Arthrobacter* probionts, two

Pseudoalteromonas phenotypes and one *Lacinutrix* phenotype, all isolated from bathed larvae, while six genotypic groups were suggested to negatively affect larval health. Putative virulence factors, i.e. haemolytic, siderophore and AHL properties, detected among four of them strengthen this view. The importance of the non-culturable microbiota in cod rearing was not covered in this study, as the focus was on probiont isolation and selection. Better understanding of the microbiota in rearing systems may contribute to successful implementation of microbial management in cod aquaculture and support the development of appropriate analytical tools.

Based on the effects of probiotics observed on larvae, it can be postulated that some inter-related mechanisms of action have occurred. Probiotic maintenance in the gut promoted a microbiota selection, a competitive exclusion of opportunistic bacteria and possibly, an indirect or direct nutritional contribution. The importance of an early probiotic treatment is stressed since maximal specific growth rate for cod is reached around 35 dph, after which a decrease in growth rate occurs (Steinarsson, 2004). Increased growth and survival at larval and juvenile rearing stages may reduce feeding costs which are the main burden of the farming industry. These results may have a great impact on the cod farming industry seeking alternative prophylactic treatments to replace the currently used chemotherapeutic agents. Shorter in-house juvenile growing time and enhanced health form the basis for sustainable cod farming.

Future perspectives

Conventional methodology applied for the search of probiotics so far is anticipated to continue in the future. Expanding molecular techniques and new knowledge on the contribution of probiotics in larval nutrition, immunomodulation, host gene expression as well as bacterial quorum quenching may well redirect the selection of criteria, making it more specific to the needs of the host. This may, in turn, contribute to a better understanding of the mechanisms of probiotic action. A better knowledge of the larval gut and rearing system microbiota may allow for the development of efficient and synergistic preventive methods, where for instance the application of prebiotics will support more easily and specifically the maintenance of desirable bacteria. Specific probiont detection and enumeration techniques will facilitate the evaluation and validation of probiotics added to rearing systems. Finally, commercialisation of probiotics will hopefully receive better positive attention by legislative bodies in order to encourage aquaculture progress and safety, leading to sustainable cod farming.

7 REFERENCES

- Abd El-Rhman AM, Khattab YAE, Shalaby AME. *Micrococcus luteus* and *Pseudomonas* species as probiotics for promoting the growth performance and health of Nile tilapia, *Oreochromis niloticus*. Fish & Shellfish Immunology 2009; 27: 175-80.
- Abee T, Krockel L, Hill C. Bacteriocins: Modes of action and potentials in food preservation and control of food poisoning. International Journal of Food Microbiology 1995; 28: 169-85.
- Abrashev IR, Genova VE, Sotirova AV, Ilieva KZ. Purification and partial characterization of neuraminidase from the non-pathogenic *Arthrobacter nicotianae*. Enzyme and Microbial Technology 1998; 22: 142-46.
- Alderson R. Effect of ammonia on the growth of juvenile Dover sole, *Solea solea* (L) and turbot, *Scophthalmus maximus* (L). Aquaculture 1979; 17: 291-309.
- Alexeeva YV, Kalinovskaya NI, Kuznetsova TA, Ivanova EP. Impact of cultivation conditions on haemolytic activity of *Pseudoalteromonas issachenkonii* KMM 3549(T). Letters in Applied Microbiology 2004; 38: 38-42.
- Alver MO, Alfredsen JA, Oie G. Estimating larval density in cod (*Gadus morhua*) first feeding tanks using measurements of feed density and larval growth rates. Aquaculture 2007; 268: 216-26.
- Aly SM, Abd-El-Rahman AM, John G, Mohamed MF. Characterization of some bacteria isolated from *Oreochromis niloticus* and their potential use as probiotics. Aquaculture 2008a; 277: 1-6.
- Aly SM, Ahmed YAG, Ghareeb AAA, Mohamed MF. Studies on *Bacillus subtilis* and *Lactobacillus acidophilus*, as potential probiotics, on the immune response and resistance of Tilapia nilotica (*Oreochromis niloticus*) to challenge infections. Fish & Shellfish Immunology 2008b; 25: 128-36.
- Amann RI, Ludwig W, Schleifer KH. Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. Microbiological Reviews 1995; 59: 143-69.
- Anonymous, (2006) Scottish Fish Farms Annual Production Survey 2005. In: <http://www.marlab.ac.uk/FRS.Web/Uploads/Documents/survey2005.pdf>. Smith RJ (ed). Aberdeen: Fisheries Research Services Marine Laboratory, pp. 48.
- Anonymous, (2009) Scottish Fish Farms Annual Production Survey 2008 In: <http://www.marlab.ac.uk/FRS.Web/Uploads/Documents/survey%20%202008version5.pdf>. Walker AJ (ed). Aberdeen: Marine Scotland Science, pp. 48.
- Aragao C, Conceicao LEC, Dinis MT, Fyhn HJ. Amino acid pools of rotifers and *Artemia* under different conditions: nutritional implications for fish larvae. Aquaculture 2004; 234: 429-45.
- Auðunsson GA, (2002) Losun og afdrif efna frá fyrirhuguðu laxeldi Samherja hf í Reyðarfirði. Mat á samlegðaráhrifum/sammögnunaráhrifum vegna byggðar og annarrar atvinnustarfsemi í Reyðarfirði, þ.m.t. hugsanlegt álver í landi Hrauns og Sómastaðargerðis í Reyðarfirði. . In: Advisory Report 02-02, June 2002 (In Icelandic). Reykjavik, Iceland: Rannsóknastofnun fiskiðnaðarins/Icelandic Fisheries Laboratories, pp. 53.
- Austin B. Biotechnology and diagnosis and control of fish pathogens. Journal of Marine Biotechnology 1998; 6: 1-2.
- Austin B, Austin D, Sutherland R, Thompson F, Swings J. Pathogenicity of vibrios to rainbow trout (*Oncorhynchus mykiss*, Walbaum) and *Artemia* nauplii. Environmental Microbiology 2005; 7: 1488-95.

- Avella MA, Gioacchini G, Decamp O, Makridis P, Bracciatelli C, Carnevali O. Application of multi-species of *Bacillus* in sea bream larviculture. *Aquaculture* 2010; 305: 12-19.
- Azad IS, Ai-Marzouk A. Autochthonous aquaculture probiotics - A critical analysis. *Research Journal of Biotechnology* 2008: 171-77.
- Bagheri T, Hedayati SA, Yavari V, Alizade M, Farzanfar A. Growth, survival and gut microbial load of rainbow trout (*Onchorhynchus mykiss*) fry given diet supplemented with probiotic during the two months of first feeding. *Turkish Journal of Fisheries and Aquatic Sciences* 2008; 8: 43-48.
- Balcazar JL, De Blas I, Ruiz-Zarzuola I, Vendrell D, Calvo AC, Marquez I, Girones O, Muzquiz JL. Changes in intestinal microbiota and humoral immune response following probiotic administration in brown trout (*Salmo trutta*). *British Journal of Nutrition* 2007; 97: 522-27.
- Barnabè G. On growing fish in intensive system. In: Barnabè G, (ed). *Aquaculture: biology and ecology of cultured species*. Ellis Horwood Series in Aquaculture and Fisheries Support, 1994: 353-56.
- Baruah K, Cam DTV, Dierckens K, Wille M, Defoirdt T, Sorgeloos P, Bossier P. *In vivo* effects of single or combined N-acyl homoserine lactone quorum sensing signals on the performance of *Macrobrachium rosenbergii* larvae. *Aquaculture* 2009; 288: 233-38.
- Baskerville-Bridges B, Kling LJ. Development and evaluation of microparticulate diets for early weaning of Atlantic cod *Gadus morhua* larvae. *Aquaculture Nutrition* 2000a; 6: 171-82.
- Baskerville-Bridges B, Kling LJ. Early weaning of Atlantic cod (*Gadus morhua*) larvae onto a microparticulate diet. *Aquaculture* 2000b; 189: 109-17.
- Baskerville-Bridges B, Kling LJ. Larval culture of Atlantic cod (*Gadus morhua*) at high stocking densities. *Aquaculture* 2000c; 181: 61-69.
- Bell JG, McEvoy LA, Estevez A, Shields RJ, Sargent JR. Optimising lipid nutrition in first-feeding flatfish larvae. *Aquaculture* 2003; 227: 211-20.
- Belser LW. Population ecology of nitrifying bacteria. *Annual Review of Microbiology* 1979; 33: 309-33.
- Benavente GP, Gatesoupe FJ. Bacteria associated with cultured rotifers and *Artemia* are detrimental to larval turbot, *Scophthalmus maximus* L *Aquacultural Engineering* 1988; 7: 289-93.
- Bergh O, (2000) Bacterial pathogens associated with early life stages of marine fish. In: 8th International Symposium in Microbial Ecology. Bell CR, Brylinsky M, Johnson-Green P (eds). Halifax, Canada: Atlantic Canada Society for Microbial Ecology, pp.
- Bergh O, Jelmert A. Iodophor disinfection of eggs of Atlantic halibut. *Journal of Aquatic Animal Health* 1996; 8: 135-45.
- Bertani G. Studies on lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli*. *Journal of Bacteriology* 1951; 62: 293-300.
- Birkbeck TH, Reid HI, Darde B, Grant AN. Activity of bronopol (Pyceze[®]) against bacteria cultured from eggs of halibut, *Hippoglossus hippoglossus* and cod, *Gadus morhua*. *Aquaculture* 2006; 254: 125-28.
- Bjornsdottir R, Johannsdottir J, Coe J, Smaradottir H, Agustsson T, Sigurgisladottir S, Gudmundsdottir BK. Survival and quality of halibut larvae (*Hippoglossus hippoglossus* L.) in intensive farming: Possible impact of the intestinal bacterial community. *Aquaculture* 2009; 286: 53-63.
- Bjornsdottir R, Karadottir EG, Johannsdottir J, Thorarinsdottir EE, Smaradottir H, Sigurgisladottir S, Gudmundsdottir BK. Selection of bacteria and the effects of

- bacterial treatment of Atlantic halibut (*Hippoglossus hippoglossus* L.) eggs and larvae. *Aquaculture* 2010; 302: 219-27.
- Björnsson B, Olafsdóttir SR. Effects of water quality and stocking density on growth performance of juvenile cod (*Gadus morhua* L.). *ICES Journal of Marine Science* 2006; 63: 326-34.
- Björnsson B, Steinarsson A. The food-unlimited growth rate of Atlantic cod (*Gadus morhua*). *Canadian Journal of Fisheries and Aquatic Sciences* 2002; 59: 494-502.
- Björnsson B, Steinarsson A, Arnason T. Growth model for Atlantic cod (*Gadus morhua*): Effects of temperature and body weight on growth rate. *Aquaculture* 2007; 271: 216-26.
- Björnsson B, Steinarsson A, Oddgeirsson M. Optimal temperature for growth and feed conversion of immature cod (*Gadus morhua* L.). *ICES Journal of Marine Science* 2001; 58: 29-38.
- Blanch AR, Alsina M, Simon M, Jofre J. Determination of bacteria associated with reared turbot (*Scophthalmus maximus*) larvae. *Journal of Applied Microbiology* 1997; 82: 729-34.
- Blom JH, Dabrowski K. Ascorbic acid metabolism in fish: Is there a maternal effect on the progeny? *Aquaculture* 1996; 147: 215-24.
- Bobe J, Labbe C. Egg and sperm quality in fish. *General and Comparative Endocrinology* 2010; 165: 535-48.
- Bogut I, Milakovic Z, Brkic S, Novoselic D, Bukvic Z. Effects of *Enterococcus faecium* on the growth rate and content of intestinal microflora in sheat fish (*Silurus glanis*). *Veterinarni Medicina* 2000; 45: 107-09.
- Bowman JP, Nichols DS. Novel members of the family Flavobacteriaceae from Antarctic maritime habitats including *Subsaximicrobium wynnwilliamsii* gen. nov., sp nov., *Subsaximicrobium saxinquilinus* sp nov., *Subsaxibacter broadyi* gen. nov., sp nov., *Lacinutrix copepodicola* gen. nov., sp nov., and novel species of the genera *Bizionia*, *Gelidibacter* and *Gillisia*. *International Journal of Systematic and Evolutionary Microbiology* 2005; 55: 1471-86.
- Bradford MM. Rapid and sensitive method for quantification of microgram quantities of protein utilizing principle of protein-dye binding. *Analytical Biochemistry* 1976; 72: 248-54.
- Brown JA, Minkoff G, Puvanendran V. Larviculture of Atlantic cod (*Gadus morhua*): progress, protocols and problems. *Aquaculture* 2003; 227: 357-72.
- Bruhn JB, Dalsgaard I, Nielsen KF, Buchholtz C, Larsen JL, Gram L. Quorum sensing signal molecules (acylated homoserine lactones) in Gram-negative fish pathogenic bacteria. *Diseases of Aquatic Organisms* 2005; 65: 43-52.
- Brunt J, Austin B. Use of a probiotic to control lactococcosis and streptococcosis in rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Journal of Fish Diseases* 2005; 28: 693-701.
- Brunt J, Newaj-Fyzul A, Austin B. The development of probiotics for the control of multiple bacterial diseases of rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Journal of Fish Diseases* 2007; 30: 573-79.
- Brunvold L, Sandaa RA, Mikkelsen H, Welde E, Bleie H, Bergh O. Characterisation of bacterial communities associated with early stages of intensively reared cod (*Gadus morhua*) using Denaturing Gradient Gel Electrophoresis (DGGE). *Aquaculture* 2007; 272: 319-27.
- Buckley LJ, Bradley TM, Allen-Guilmette J. Production, quality, and low temperature incubation of eggs of Atlantic cod *Gadus morhua* and haddock *Melanogrammus aeglefinus* in captivity. *Journal of the World Aquaculture Society* 2000; 31: 22-29.
- Cahill MM. Bacterial flora of fishes - A review. *Microbial Ecology* 1990; 19: 21-41.

- Cahu C, Infante JZ. Substitution of live food by formulated diets in marine fish larvae. *Aquaculture* 2001; 200: 161-80.
- Cahu CL, Gisbert E, Villeneuve LAN, Morais S, Hamza N, Wold PA, Infante JLZ. Influence of dietary phospholipids on early ontogenesis of fish. *Aquaculture Research* 2009; 40: 989-99.
- Caipang CMA, Brinchmann MF, Kiron V. Antagonistic activity of bacterial isolates from intestinal microbiota of Atlantic cod, *Gadus morhua*, and an investigation of their immunomodulatory capabilities. *Aquaculture Research* 2010a; 41: 249-56.
- Caipang CMA, Kulkarni A, Brinchmann MF, Korsnes K, Kiron V. Detection of *Francisella piscicida* in Atlantic cod (*Gadus morhua* L) by the loop-mediated isothermal amplification (LAMP) reaction. *Veterinary Journal* 2010b; 184: 357-61.
- Callan C, Jordaan A, Kling LJ. Reducing *Artemia* use in the culture of Atlantic cod (*Gadus morhua*). *Aquaculture* 2003; 219: 585-95.
- Carnevali O, de Vivo L, Sulpizio R, Olivetto I, Silvi S, Cresci A. Growth improvement by probiotic in European sea bass juveniles (*Dicentrarchus labrax*, L.), with particular attention to IGF-1, myostatin and cortisol gene expression. *Aquaculture* 2006; 258: 430-38.
- Carnevali O, Zamponi MC, Sulpizio R, Rollo A, Nardi M, Orpianesi C, Silvi S, Caggiano M, Polzonetti AM, Cresci A. Administration of probiotic strain to improve sea bream wellness during development. *Aquaculture International* 2004; 12: 377-86.
- Cha C, Gao P, Chen YC, Shaw PD, Farrand SK. Production of acyl-homoserine lactone quorum-sensing signals by gram-negative plant-associated bacteria. *Molecular Plant-Microbial Interactions* 1998; 11: 1119-29.
- Chabrillon M, Arijo S, Diaz-Rosales P, Balebona MC, Morinigo MA. Interference of *Listonella anguillarum* with potential probiotic microorganisms isolated from farmed gilthead seabream (*Sparus aurata*, L.). *Aquaculture Research* 2006; 37: 78-86.
- Chang CI, Liu WY. An evaluation of two probiotic bacterial strains, *Enterococcus faecium* SF68 and *Bacillus toyoi*, for reducing edwardsiellosis in cultured European eel, *Anguilla anguilla* L. *Journal of Fish Diseases* 2002; 25: 311-15.
- Colwell RR, Brayton PR, Grimes DJ, Roszak DB, Huq SA, Palmer LM. Viable but non-culturable *Vibrio cholerae* and related pathogens in the environment: Implications for release of genetically engineered microorganisms. *Biotechnology* 1985; 3: 817-20.
- Comes LC, Brinn RP, Marcon JL, Dantas LA, Brandao FR, de Abreu JS, McComb DM, Baldisserotto B. Using Efinol® L during transportation of marbled hatchetfish, *Carnegiella strigata* (Gunther). *Aquaculture Research* 2008; 39: 1292-98.
- Conceicao LEC, Aragao C, Richard N, Engrola S, Gavaia P, Mira S, Dias J. Novel methodologies in marine fish larval nutrition. *Fish Physiology and Biochemistry* 2010; 36: 1-16.
- Conceicao LEC, Skjermo J, Skjak-Braek G, Verreth JAJ. Effect of an immunostimulating alginate on protein turnover of turbot (*Scophthalmus maximus* L.) larvae. *Fish Physiology and Biochemistry* 2001; 24: 207-12.
- Czajkowski R, Jafra S. Quenching of acyl-homoserine lactone-dependent quorum sensing by enzymatic disruption of signal molecules. *Acta Biochimica Polonica* 2009; 56: 1-16.
- Dabrowski K, Ciereszko A. Ascorbic acid and reproduction in fish: endocrine regulation and gamete quality. *Aquaculture Research* 2001; 32: 623-38.
- Dahllöf I, Baillie H, Kjelleberg S. *rpoB*-based microbial community analysis avoids limitations inherent in 16S rRNA gene intraspecies heterogeneity. *Applied and Environmental Microbiology* 2000; 66: 3376-80.

- Dalgaard P, Mejholm O, Christiansen TJ, Huss HH. Importance of *Photobacterium phosphoreum* in relation to spoilage of modified atmosphere-packed fish products. *Letters in Applied Microbiology* 1997; 24: 373-78.
- Dang TVC, Dinh TN, Ceuppens S, Nguyen VH, Dierckens K, Wille M, Sorgeloos P, Bossier P. Effect of N-acyl homoserine lactone-degrading enrichment cultures on *Macrobrachium rosenbergii* larviculture. *Aquaculture* 2009; 294: 5-13.
- Davidson CM, Cronin F. Medium for selective enumeration of lactic acid bacteria from foods. *Applied Microbiology* 1973; 26: 439-40.
- Decamp O, Moriarty DJW, Lavens P. Probiotics for shrimp larviculture: review of field data from Asia and Latin America. *Aquaculture Research* 2008; 39: 334-38.
- Dhert P, Lavens P, Sorgeloos P. Stress evaluation: a tool for quality control of hatchery produced shrimp and fish fry. *Aquaculture Europe* 1992; 17: 6-10.
- Dixon M, Webb EC. *Enzymes, 3rd edition*. London: Longman Group Ltd; 1979: 207-973.
- Eddy SD, Jones SH. Microbiology of summer flounder *Paralichthys dentatus* fingerling production at a marine fish hatchery. *Aquaculture* 2002; 211: 9-28.
- El-Haroun ER, Goda A, Chowdhury MAK. Effect of dietary probiotic Biogen^(R) supplementation as a growth promoter on growth performance and feed utilization of Nile tilapia *Oreochromis niloticus* (L.). *Aquaculture Research* 2006; 37: 1473-80.
- El-Shafai SA, El-Gohary FA, Nasr FA, van der Steen NP, Gijzen HJ. Chronic ammonia toxicity to duckweed-fed tilapia (*Oreochromis niloticus*). *Aquaculture* 2004; 232: 117-27.
- Ellis AE. Innate host defense mechanisms of fish against viruses and bacteria. *Developmental and Comparative Immunology* 2001; 25: 827-39.
- Engelsen AR, Sandlund N, Fiksdal IU, Bergh O. Immunohistochemistry of Atlantic cod larvae *Gadus morhua* experimentally challenged with *Vibrio anguillarum*. *Diseases of Aquatic Organisms* 2008; 80: 13-20.
- Escaffre AM, Bazin D, Bergot P. Disinfection of *Sparus aurata* eggs with glutaraldehyde. *Aquaculture International* 2001; 9: 451-58.
- Falk-Petersen IB. Comparative organ differentiation during early life stages of marine fish. *Fish & Shellfish Immunology* 2005; 19: 397-412.
- FAO, (2009) The state of world fisheries and aquaculture. In. Department FFaA (ed). Rome: Food and Agriculture Organization of the United Nations, pp. 178.
- Finn RN, Ronnestad I, van der Meeren T, Fyhn HJ. Fuel and metabolic scaling during the early life stages of Atlantic cod *Gadus morhua*. *Marine Ecology-Progress Series* 2002; 243: 217-34.
- Fitzsimmons SD, Perutz M. Effects of egg incubation temperature on survival, prevalence and types of malformations in vertebral column of Atlantic Cod (*Gadus morhua*) larvae. *Bulletin of the European Association of Fish Pathologists* 2006; 26: 80-86.
- Fjellheim AJ, Klinkenberg G, Skjermo J, Aasen IM, Vadstein O. Selection of candidate probiotics by two different screening strategies from Atlantic cod (*Gadus morhua* L.) larvae. *Veterinary Microbiology* 2010; 144: 153-59.
- Fjellheim AJ, Playfoot KJ, Skjermo J, Vadstein O. Vibrionaceae dominates the microflora antagonistic towards *Listonella anguillarum* in the intestine of cultured Atlantic cod (*Gadus morhua* L.) larvae. *Aquaculture* 2007; 269: 98-106.
- Fuller R. Probiotics in man and animals. *Journal of Applied Bacteriology* 1989; 66: 365-78.
- Gao HW, Li FH, Zhang XJ, Wang B, Xiang JH. Rapid, sensitive detection of *Vibrio anguillarum* using loop-mediated isothermal amplification. *Chinese Journal of Oceanology and Limnology* 2010; 28: 62-66.

- Garcia AS, Parrish CC, Brown JA. Use of enriched rotifers and *Artemia* during larviculture of Atlantic cod (*Gadus morhua* Linnaeus, 1758): effects on early growth, survival and lipid composition. *Aquaculture Research* 2008; 39: 406-19.
- Gatesoupe FJ. The effect of 3 strains of lactic bacteria on the production rate of rotifers, *Brachionus plicatilis*, and their dietary value for larval turbot, *Scophthalmus maximus*. *Aquaculture* 1991; 96: 335-42.
- Gatesoupe FJ. Lactic acid bacteria increase the resistance of turbot larvae, *Scophthalmus maximus*, against pathogenic *Vibrio*. *Aquatic Living Resources* 1994; 7: 277-82.
- Gatesoupe FJ. Siderophore production and probiotic effect of *Vibrio* sp. associated with turbot larvae, *Scophthalmus maximus*. *Aquatic Living Resources* 1997; 10: 239-46.
- Gatesoupe FJ. The use of probiotics in aquaculture. *Aquaculture* 1999; 180: 147-65.
- Gatesoupe FJ. Probiotic and formaldehyde treatments of *Artemia* nauplii as food for larval pollack, *Pollachius pollachius*. *Aquaculture* 2002; 212: 347-60.
- Gatesoupe FJ. Live yeasts in the gut: Natural occurrence, dietary introduction, and their effects on fish health and development. *Aquaculture* 2007; 267: 20-30.
- Gatesoupe FJ. Updating the importance of lactic acid bacteria in fish farming: Natural occurrence and probiotic treatments. *Journal of Molecular Microbiology and Biotechnology* 2008; 14: 107-14.
- Gatesoupe FJ, Lambert C, Nicolas JL. Pathogenicity of *Vibrio splendidus* strains associated with turbot larvae, *Scophthalmus maximus*. *Journal of Applied Microbiology* 1999; 87: 757-63.
- Ghosh S, Sinha A, Sahu C. Dietary probiotic supplementation in growth and health of live-bearing ornamental fishes. *Aquaculture Nutrition* 2008; 14: 289-99.
- Gildberg A, Mikkelsen H. Effects of supplementing the feed to Atlantic cod (*Gadus morhua*) fry with lactic acid bacteria and immuno-stimulating peptides during a challenge trial with *Vibrio anguillarum*. *Aquaculture* 1998; 167: 103-13.
- Gildberg A, Mikkelsen H, Sandaker E, Ringo E. Probiotic effect of lactic acid bacteria in the feed on growth and survival of fry of Atlantic cod (*Gadus morhua*). *Hydrobiologia* 1997; 352: 279-85.
- Gomes LC, Brinn RP, Marcon JL, Dantas LA, Brandao FR, de Abreu JS, Lemos PEM, McComb DM, Baldisserotto B. Benefits of using the probiotic Efinol® L during transportation of cardinal tetra, *Paracheirodon axelrodi* (Schultz), in the Amazon. *Aquaculture Research* 2009; 40: 157-65.
- Gomez-Gil B, Thompson FL, Thompson CC, Swings J. *Vibrio pacinii* sp nov., from cultured aquatic organisms. *International Journal of Systematic and Evolutionary Microbiology* 2003; 53: 1569-73.
- Gorman SP, Scott EM, Russell AD. A review - Antimicrobial activity, uses and mechanism of action of glutaraldehyde. *Journal of Applied Bacteriology* 1980; 48: 161-90.
- Gram L, Melchiorson J, Spanggaard B, Huber I, Nielsen TF. Inhibition of *Vibrio anguillarum* by *Pseudomonas fluorescens* AH2, a possible probiotic treatment of fish. *Applied and Environmental Microbiology* 1999; 65: 969-73.
- Griffiths S, Melville K, Cook M, Vincent S, St Pierre M, Lanteigne C. Profiling of bacterial species associated with haddock larviculture by PCR amplification of 16S rDNA and denaturing gradient gel electrophoresis. *Journal of Aquatic Animal Health* 2001; 13: 355-63.
- Grisez L, Reyniers J, Verdonck L, Swings J, Ollevier F. Dominant intestinal microflora of sea bream and sea bass larvae, from two hatcheries, during larval development. *Aquaculture* 1997; 155: 387-99.

- Grotmol S, Dahl-Paulsen E, Totland GK. Hatchability of eggs from Atlantic cod, turbot and Atlantic halibut after disinfection with ozonated seawater. *Aquaculture* 2003; 221: 245-54.
- Guan LL, Onuki H, Kamino K. Bacterial growth stimulation with exogenous siderophore and synthetic N-acyl homoserine lactone autoinducers under iron-limited and low-nutrient conditions. *Applied and Environmental Microbiology* 2000; 66: 2797-803.
- Gudmundsdottir BK, Bjornsdottir B. Vaccination against atypical furunculosis and winter ulcer disease of fish. *Vaccine* 2007; 25: 5512-23.
- Hamre K. Nutrition in cod (*Gadus morhua*) larvae and juveniles. *ICES Journal of Marine Science* 2006; 63: 267-74.
- Hamre K, Mollan TA, Saele O, Erstad B. Rotifers enriched with iodine and selenium increase survival in Atlantic cod (*Gadus morhua*) larvae. *Aquaculture* 2008a; 284: 190-95.
- Hamre K, Srivastava A, Ronnestad I, Mangor-Jensen A, Stoss J. Several micronutrients in the rotifer *Brachionus* sp may not fulfil the nutritional requirements of marine fish larvae. *Aquaculture Nutrition* 2008b; 14: 51-60.
- Hansen GH, Olafsen JA. Bacterial colonization of cod (*Gadus morhua* L) and halibut (*Hippoglossus hippoglossus*) eggs in marine aquaculture. *Applied and Environmental Microbiology* 1989; 55: 1435-46.
- Hansen GH, Olafsen JA. Bacterial interactions in early life stages of marine cold water fish. *Microbial Ecology* 1999; 38: 1-26.
- Hargreaves JA. Nitrogen biogeochemistry of aquaculture ponds. *Aquaculture* 1998; 166: 181-212.
- Hentschel U, Schmid M, Wagner M, Fieseler L, Gernert C, Hacker J. Isolation and phylogenetic analysis of bacteria with antimicrobial activities from the Mediterranean sponges *Aplysina aerophoba* and *Aplysina cavernicola*. *FEMS Microbiology Ecology* 2001; 35: 305-12.
- Hjelm M, Bergh O, Riaza A, Nielsen J, Melchiorson J, Jensen S, Duncan H, Ahrens P, Birkbeck H, Gram L. Selection and identification of autochthonous potential probiotic bacteria from turbot larvae (*Scophthalmus maximus*) rearing units. *Systematic and Applied Microbiology* 2004; 27: 360-71.
- Hoang TT, Schweizer HP. Characterization of *Pseudomonas aeruginosa* Enoyl-Acyl carrier protein reductase (FabI): a target for the antimicrobial triclosan and its role in acylated homoserine lactone synthesis. *Journal of Bacteriology* 1999; 181: 5489-97.
- Hoj L, Bourne DG, Hall MR. Localization, abundance and community structure of bacteria associated with *Artemia*: Effects of nauplii enrichment and antimicrobial treatment. *Aquaculture* 2009; 293: 278-85.
- Howarth RS, Sprague JB. Copper lethality to rainbow trout in waters of various hardness and pH. *Water Research* 1978; 12: 455-62.
- Huang CC, Liao SM, Tsai WC, Wang HH. Using direct epifluorescent microscopic count for rapid enumeration of viable yeast and bacteria in injured conditions. *Journal of Food and Drug Analysis* 2005; 13: 143-50.
- Imsland AK, Foss A, Koedijk R, Folkvord A, Stefansson SO, Jonassen TM. Short- and long-term differences in growth, feed conversion efficiency and deformities in juvenile Atlantic cod (*Gadus morhua*) started on rotifers or zooplankton. *Aquaculture Research* 2006; 37: 1015-27.
- Irianto A, Austin B. Use of probiotics to control furunculosis in rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Journal of Fish Diseases* 2002; 25: 333-42.
- Isnansetyo A, Istiqomah I, Muhtadi, Sinansari S, Hernawan RK, Triyanto, Widada J. A potential bacterial biocontrol agent, strain S2V2 against pathogenic marine *Vibrio* in aquaculture. *World Journal of Microbiology & Biotechnology* 2009; 25: 1103-13.

- Ivanova EP, Flavier S, Christen R. Phylogenetic relationships among marine *Alteromonas*-like proteobacteria: emended description of the family Alteromonadaceae and proposal of Pseudoalteromonadaceae fam. nov., Colwelliaceae fam. nov., Shewanellaceae fam. nov., Montellaceae fam. nov., Ferrimonadaceae fam. nov., Idiomarinaceae fam. nov and Psychromonadaceae fam. nov. *International Journal of Systematic and Evolutionary Microbiology* 2004; 54: 1773-88.
- Izquierdo MS, Fernandez-Palacios H, Tacon AGJ. Effect of broodstock nutrition on reproductive performance of fish. *Aquaculture* 2001; 197: 25-42.
- Jatoba A, Vieira FD, Neto CB, Silva BC, Mourino JLP, Jeronimo GT, Dotta G, Martins ML. Lactic acid bacteria isolated from the intestinal tract of Nile tilapia utilized as probiotic. *Pesquisa Agropecuaria Brasileira* 2008; 43: 1201-07.
- Jay JM. *Modern Food Microbiology*. New York, USA: Van Nostrand Reinhold; 1992: 701.
- Jayanth K, Jeyasekaran G, Shakila RJ. Biocontrol of fish bacterial pathogens by the antagonistic bacteria isolated from the coastal waters of Gulf of Mannar, India. *Bulletin of the European Association of Fish Pathologists* 2001; 21: 12-18.
- Jensen S, Ovreas L, Bergh O, Torsvik V. Phylogenetic analysis of bacterial communities associated with larvae of the Atlantic halibut propose succession from a uniform normal flora. *Systematic and Applied Microbiology* 2004; 27: 728-36.
- Joborn A, Olsson JC, Westerdahl A, Conway PL, Kjelleberg S. Colonization in the fish intestinal tract and production of inhibitory substances in intestinal mucus and faecal extracts by *Carnobacterium* sp. strain K1. *Journal of Fish Diseases* 1997; 20: 383-92.
- Jónsson G, (2010) Ársskýrsla dýralæknis fisksjúkdóma 2009. In. <http://www.mast.is/Uploads/document/arsskyrsla/arskyrsladyralaeknisfisksjukdoma2009.pdf> (ed). Selfoss: Matvælastofnun, pp. 20.
- Jordaan A, Brown JA. The risk of running on empty: the influence of age on starvation and gut fullness in larval Atlantic cod (*Gadus morhua*). *Canadian Journal of Fisheries and Aquatic Sciences* 2003; 60: 1289-98.
- Kamler E. Resource allocation in yolk-feeding fish. *Reviews in Fish Biology and Fisheries* 2008; 18: 143-200.
- Kent AD, Smith DJ, Benson BJ, Triplett EW. Web-based phylogenetic assignment tool for analysis of terminal restriction fragment length polymorphism profiles of microbial communities. *Applied and Environmental Microbiology* 2003; 69: 6768-76.
- Kjorsvik E, Hoehne-Reitan K, Reitan KI. Egg and larval quality criteria as predictive measures for juvenile production in turbot (*Scophthalmus maximus* L.). *Aquaculture* 2003; 227: 9-20.
- Korsnes K, Nicolaisen O, Skar CK, Nerland AH, Bergh O. Bacteria in the gut of juvenile cod *Gadus morhua* fed live feed enriched with four different commercial diets. *ICES Journal of Marine Science* 2006; 63: 296-301.
- Kulkarni A, Caipang CMA, Brinchmann MF, Korsnes K, Kiron V. Loop-mediated isothermal amplification - An assay for the detection of atypical furunculosis caused by *Aeromonas salmonicida* in Atlantic cod, *Gadus morhua*. *Journal of Rapid Methods and Automation in Microbiology* 2009; 17: 476-89.
- Kvale A, Harboe T, Mangor-Jensen A, Hamre K. Effects of protein hydrolysate in weaning diets for Atlantic cod (*Gadus morhua* L.) and Atlantic halibut (*Hippoglossus hippoglossus* L.). *Aquaculture Nutrition* 2009; 15: 218-27.
- Kvale A, Mangor-Jensen A, Moren M, Espe M, Hamre K. Development and characterisation of some intestinal enzymes in Atlantic cod (*Gadus morhua* L.) and Atlantic halibut (*Hippoglossus hippoglossus* L.) larvae. *Aquaculture* 2007; 264: 457-68.

- Langdon C. Microparticle types for delivering nutrients to marine fish larvae. *Aquaculture* 2003; 227: 259-75.
- Lange S, Dodds AW, Gudmundsdottir S, Bambir SH, Magnadottir B. The ontogenic transcription of complement component C3 and apolipoprotein A-I tRNA in Atlantic cod (*Gadus morhua* L.) - a role in development and homeostasis? *Developmental and Comparative Immunology* 2005; 29: 1065-77.
- Lange S, Dodds AW, Magnadottir B. Isolation and characterization of complement component C3 from Atlantic cod (*Gadus morhua* L.) and Atlantic halibut (*Hippoglossus hippoglossus* L.). *Fish & Shellfish Immunology* 2004a; 16: 227-39.
- Lange SR, Bambir S, Dodds AW, Magnadottir B. The ontogeny of complement component C3 in Atlantic cod (*Gadus morhua* L.) - an immunohistochemical study. *Fish & Shellfish Immunology* 2004b; 16: 359-67.
- Lee SO, Kato J, Nakashima K, Kuroda A, Ikeda T, Takiguchi N, Ohtake H. Cloning and characterization of extracellular metal protease gene of the algicidal marine bacterium *Pseudoalteromonas* sp strain A28. *Bioscience Biotechnology and Biochemistry* 2002; 66: 1366-69.
- Léssel R. Thermal effect on bacterial flora in the gut of rainbow trout and African catfish. *In*: Léssel R, (ed). *Microbiology of Poecilotherms*. Amsterdam: Elsevier, 1990: 33-38.
- Lezama-Cervantes C, Paniagua-Michel JD, Zamora-Castro J. Bioremediation of effluents ones of the culture of *Litopenaeus vannamei* (Boone, 1931) using microbial mats in a recirculating system. *Latin American Journal of Aquatic Research* 2010; 38: 129-41.
- Li JQ, Tan BP, Mai K, Ai QH, Zhang WB, Xu W, Liufu ZG, Ma HM. Comparative study between probiotic bacterium *Arthrobacter* XE-7 and chloramphenicol on protection of *Penaeus chinensis* post-larvae from pathogenic vibrios. *Aquaculture* 2006; 253: 140-47.
- Lin HZ, Guo ZX, Yang YY, Zheng WH, Li ZJJ. Effect of dietary probiotics on apparent digestibility coefficients of nutrients of white shrimp *Litopenaeus vannamei* Boone. *Aquaculture Research* 2004; 35: 1441-47.
- Lisle JT, Hamilton MA, Willse AR, McFeters GA. Comparison of fluorescence microscopy and solid-phase cytometry methods for counting bacteria in water. *Applied and Environmental Microbiology* 2004; 70: 5343-48.
- Liu F, Han WY. Reuse strategy of wastewater in prawn nursery by microbial remediation. *Aquaculture* 2004; 230: 281-96.
- Longeon A, Peduzzi J, Barthelemy M, Corre S, Nicolas JL, Guyot M. Purification and partial identification of novel antimicrobial protein from marine bacterium *Pseudoalteromonas* species strain x 4 53. *Marine Biotechnology* 2004; 6: 633-41.
- Lopez-Caballero ME, Goncalves A, Nunes ML. Effect of CO₂/O₂-containing modified atmosphere on packed deepwater shrimp (*Parapenaeus longirostris*). *European Food Research and Technology* 2002; 214: 192-97.
- Lopez-Torres MA, Lizarraga-Partida ML. Bacteria isolated on TCBS media associated with hatched *Artemia* cysts of commercial brands. *Aquaculture* 2001; 194: 11-20.
- Macey BM, Coyne VE. Colonization of the gastrointestinal tract of the farmed South African abalone *Haliotis midae* by the probionts *Vibrio midae* SY9, *Cryptococcus* sp SS1, and *Debaryomyces hansenii* AY1. *Marine Biotechnology* 2006; 8: 246-59.
- Maeda T, Matsuo Y, Furushita M, Shiba T. Seasonal dynamics in a coastal *Vibrio* community examined by a rapid clustering method based on 16S rDNA. *Fisheries Science* 2003; 69: 385-94.
- Magnadottir B. Comparison of immunoglobulin (IgM) from four fish species. *Icelandic Agricultural Sciences* 1998; 12: 47-59.

- Magnadottir B. Innate immunity of fish (overview). *Fish & Shellfish Immunology* 2006; 20: 137-51.
- Magnadottir B. Immunological control of fish diseases. *Marine Biotechnology* 2010; 12: 361-79.
- Magnadottir B, Gudmundsdottir BK, Lange S, Steinarsson A, Oddgeirsson M, Bowden T, Bricknell I, Dalmo RA, Gudmundsdottir S. Immunostimulation of larvae and juveniles of cod, *Gadus morhua* L. *Journal of Fish Diseases* 2006; 29: 147-55.
- Magnadottir B, Jonsdottir H, Helgason S, Bjornsson B, Jorgensen TO, Pilstrom L. Humoral immune parameters in Atlantic cod (*Gadus morhua* L.) - I. The effects of environmental temperature. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology* 1999; 122: 173-80.
- Magnadottir B, Lange S. Is Apolipoprotein A-I a regulating protein for the complement system of cod (*Gadus morhua* L.)? *Fish & Shellfish Immunology* 2004; 16: 265-69.
- Magnadottir B, Lange S, Gudmundsdottir S, Bogwald J, Dalmo RA. Ontogeny of humoral immune parameters in fish. *Fish & Shellfish Immunology* 2005; 19: 429-39.
- Magnadottir B, Lange S, Steinarsson A, Gudmundsdottir S. The ontogenic development of innate immune parameters of cod (*Gadus morhua* L.). *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology* 2004; 139: 217-24.
- Makridis P, Jon Fjellheim A, Skjermo J, Vadstein O. Colonization of the gut in first feeding turbot by bacterial strains added to the water or bioencapsulated in rotifers. *Aquaculture International* 2000; 8: 367-80.
- Makridis P, Martins S, Reis J, Dinis MT. Use of probiotic bacteria in the rearing of Senegalese sole (*Solea senegalensis*) larvae. *Aquaculture Research* 2008; 39: 627-34.
- Makridis P, Martins S, Vercauteren T, Van Driessche K, Decamp O, Dinis MT. Evaluation of candidate probiotic strains for gilthead sea bream larvae (*Sparus aurata*) using an *in vivo* approach. *Letters of Applied Microbiology* 2005; 40: 274-77.
- Makridis P, Moreira C, Costa RA, Rodrigues P, Dinis MT. Use of microalgae bioencapsulated in *Artemia* during the weaning of Senegalese sole (*Solea senegalensis* Kaup). *Aquaculture* 2009; 292: 153-57.
- Manefield M, Rasmussen TB, Henzter M, Andersen JB, Steinberg P, Kjelleberg S, Givskov M. Halogenated furanones inhibit quorum sensing through accelerated LuxR turnover. *Microbiology-SGM* 2002; 148: 1119-27.
- Marteinsdottir G, Steinarsson A. Maternal influence on the size and viability of Iceland cod *Gadus morhua* eggs and larvae. *Journal of Fish Biology* 1998; 52: 1241-58.
- McClean KH, Winson MK, Fish L, Taylor A, Chhabra SR, Camara M, Daykin M, Lamb JH, Swift S, Bycroft BW, Stewart G, Williams P. Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of N-acylhomoserine lactones. *Microbiology-SGM* 1997; 143: 3703-11.
- McIntosh D, Ji B, Forward BS, Puvanendran V, Boyce D, Ritchie R. Culture-independent characterization of the bacterial populations associated with cod (*Gadus morhua* L.) and live feed at an experimental hatchery facility using denaturing gradient gel electrophoresis. *Aquaculture* 2008; 275: 42-50.
- McKenzie DJ, Shingles A, Taylor EW. Sub-lethal plasma ammonia accumulation and the exercise performance of salmonids. *Comparative Biochemistry and Physiology Part A-Molecular & Integrative Physiology* 2003; 135: 515-26.
- Merrifield DL, Burnard D, Bradley G, Davies SJ, Baker RTM. Microbial community diversity associated with the intestinal mucosa of farmed rainbow trout (*Oncorhynchus mykiss* Walbaum). *Aquaculture Research* 2009; 40: 1064-72.

- Merrifield DL, Dimitroglou A, Foey A, Davies SJ, Baker RTM, Bogwald J, Castex M, Ringo E. The current status and future focus of probiotic and prebiotic applications for salmonids. *Aquaculture* 2010; 302: 1-18.
- Metivier A, Pilet MF, Dousset X, Sorokine O, Anglade P, Zagorec M, Piard JC, Marion D, Cenatiempo Y, Fremaux C. Divercin V41, a new bacteriocin with two disulphide bonds produced by *Carnobacterium divergens* V41: primary structure and genomic organization. *Microbiology-Uk* 1998; 144: 2837-44.
- Miyamoto K, Tsujibo H, Nukui E, Itoh H, Kaidzu Y, Inamori Y. Isolation and characterization of the genes encoding two metalloproteases (MprI and MprII) from a marine bacterium, *Alteromonas* sp strain O-7. *Bioscience Biotechnology and Biochemistry* 2002; 66: 416-21.
- Moe MAJ. *The Marine Aquarium Reference: Systems and Invertebrates*. Plantation, FL, USA: Green Turtle Publications; 1993: 510.
- Monk J, Puvanendran V, Brown JA. Do different light regimes affect the foraging behaviour, growth and survival of larval cod (*Gadus morhua* L.)? *Aquaculture* 2006; 257: 287-93.
- Moriarty DJW. Control of luminous *Vibrio* species in penaeid aquaculture ponds. *Aquaculture* 1998; 164: 351-58.
- Munro PD, Barbour A, Birkbeck TH. Comparison of the growth and survival of larval turbot in the absence of culturable bacteria with those in the presence of *Vibrio anguillarum*, *Vibrio alginolyticus*, or a marine *Aeromonas* sp. *Applied and Environmental Microbiology* 1995; 61: 4425-28.
- Muyzer G, Dewaal EC, Uitterlinden AG. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S ribosomal RNA. *Applied and Environmental Microbiology* 1993; 59: 695-700.
- Nakase G, Nakagawa Y, Miyashita S, Nasu T, Senoo S, Matsubara H, Eguchi M. Association between bacterial community structures and mortality of fish larvae in intensive rearing systems. *Fisheries Science* 2007; 73: 784-91.
- Navarrete P, Magne F, Mardones P, Riveros M, Opazo R, Suau A, Pochart P, Romero J. Molecular analysis of intestinal microbiota of rainbow trout (*Oncorhynchus mykiss*). *FEMS Microbiology Ecology* 2010; 71: 148-56.
- NDF, (2010) Aquaculture statistics - Cod. <http://www.fiskeridir.no/english/statistics/norwegian-aquaculture/aquaculture-statistics/cod>, retrieved 13.06.2010.
- Nealson KH, Wimpee B, Wimpee C. Identification of *Vibrio splendidus* as a member of the planktonic luminous bacteria from the Persian Gulf and Kuwait region with LuxA probes *Applied and Environmental Microbiology* 1993; 59: 2684-89.
- Ng WK, Koh CB, Sudesh K, Siti-Zahrah A. Effects of dietary organic acids on growth, nutrient digestibility and gut microflora of red hybrid tilapia, *Oreochromis* sp., and subsequent survival during a challenge test with *Streptococcus agalactiae*. *Aquaculture Research* 2009; 40: 1490-500.
- Nikoskelainen S, Ouwehand A, Salminen S, Bylund G. Protection of rainbow trout (*Oncorhynchus mykiss*) from furunculosis by *Lactobacillus rhamnosus*. *Aquaculture* 2001; 198: 229-36.
- Nikoskelainen S, Ouwehand AC, Bylund G, Salminen S, Lilius EM. Immune enhancement in rainbow trout (*Oncorhynchus mykiss*) by potential probiotic bacteria (*Lactobacillus rhamnosus*). *Fish & Shellfish Immunology* 2003; 15: 443-52.
- Njoroge J, Sperandio V. Jamming bacterial communication: New approaches for the treatment of infectious diseases. *EMBO Molecular Medicine* 2009; 1: 201-10.

- O'Brien-MacDonald K, Brown JA, Parrish CC. Growth, behaviour, and digestive enzyme activity in larval Atlantic cod (*Gadus morhua*) in relation to rotifer lipid. ICES Journal of Marine Science 2006; 63: 275-84.
- Oie G, Makridis P, Reitan KI, Olsen Y. Protein and carbon utilization of rotifers (*Brachionus plicatilis*) in first feeding of turbot larvae (*Scophthalmus maximus* L). Aquaculture 1997; 153: 103-22.
- Oie G, Reitan KI, Olsen Y. Comparison of rotifer culture quality with yeast plus oil and algal-based cultivation diets. Aquaculture International 1994; 2: 225-38.
- Olafsen JA. Interactions between fish larvae and bacteria in marine aquaculture. Aquaculture 2001; 200: 223-47.
- Olsen AI, Olsen Y, Attramadal Y, Christie K, Birkbeck TH, Skjermo J, Vadstein O. Effects of short term feeding of microalgae on the bacterial flora associated with juvenile *Artemia franciscana*. Aquaculture 2000; 190: 11-25.
- Orozco-Medina C, Maeda-Martinez AM, Lopez-Cortes A. Effect of aerobic Gram-positive heterotrophic bacteria associated with *Artemia franciscana* cysts on the survival and development of its larvae. Aquaculture 2002; 213: 15-29.
- Otterlei E, Nyhammer G, Folkvord A, Stefansson SO. Temperature- and size-dependent growth of larval and early juvenile Atlantic cod (*Gadus morhua*): a comparative study of Norwegian coastal cod and northeast Arctic cod. Canadian Journal of Fisheries and Aquatic Sciences 1999; 56: 2099-111.
- Ottesen OH, Olafsen JA. Effects on survival and mucous cell proliferation of Atlantic halibut, *Hippoglossus hippoglossus* L., larvae following microflora manipulation. Aquaculture 2000; 187: 225-38.
- Paisly LG, Ariel E, Lyngstad T, Jónsson G, Vennerstöm P, Hellström A, Ostergaard P. An overview of aquaculture in the Nordic countries. Journal of the World Aquaculture Society 2010; 41: 1-17.
- Panigrahi A, Azad IS. Microbial intervention for better fish health in aquaculture: The Indian scenario. Fish Physiology and Biochemistry 2007; 33: 429-40.
- Panigrahi A, Kiron V, Kobayashi T, Puangkaew J, Satoh S, Sugita H. Immune responses in rainbow trout *Oncorhynchus mykiss* induced by a potential probiotic bacteria *Lactobacillus rhamnosus* JCM 1136. Veterinary Immunology and Immunopathology 2004; 102: 379-88.
- Panigrahi A, Kiron V, Puangkaew J, Kobayashi T, Satoh S, Sugita H. The viability of probiotic bacteria as a factor influencing the immune response in rainbow trout *Oncorhynchus mykiss*. Aquaculture 2005; 243: 241-54.
- Panigrahi A, Kiron V, Satoh S, Hirono I, Kobayashi T, Sugita H, Puangkaew J, Aoki T. Immune modulation and expression of cytokine genes in rainbow trout *Oncorhynchus mykiss* upon probiotic feeding. Developmental and Comparative Immunology 2007; 31: 372-82.
- Park HG, Puvanendran V, Kellett A, Parrish CC, Brown JA. Effect of enriched rotifers on growth, survival, and composition of larval Atlantic cod (*Gadus morhua*). ICES Journal of Marine Science 2006; 63: 285-95.
- Peck MA, Buckley LJ. Measurements of larval Atlantic cod (*Gadus morhua*) routine metabolism: temperature effects, diel differences and individual-based modeling. Journal of Applied Ichthyology 2008; 24: 144-49.
- Peck MA, Buckley LJ, O'Bryan LM, Davies EJ, Lapolla AE. Efficacy of egg surface disinfectants in captive spawning Atlantic cod *Gadus morhua* L. and haddock *Melanogrammus aeglefinus* L. Aquaculture Research 2004; 35: 992-96.

- Pedersen GM, Gildberg A, Olsen RL. Effects of including cationic proteins from cod milt in the feed to Atlantic cod (*Gadus morhua*) fry during a challenge trial with *Vibrio anguillarum*. *Aquaculture* 2004; 233: 31-43.
- Pedersen T, Falk-Petersen IB. Morphological changes during metamorphosis in cod (*Gadus morhua* L.), with particular reference to the development of the stomach and pyloric ceca. *Journal of Fish Biology* 1992; 41: 449-61.
- Penney RW, Lush PL, Wade AJ, Brown JA, Burton MPM. Effect of photoperiod manipulation on broodstock spawning, fertilization success, and egg developmental abnormalities in Atlantic cod, *Gadus morhua*. *Journal of the World Aquaculture Society* 2006a; 37: 273-81.
- Penney RW, Lush PL, Wade J, Brown JA, Parrish CC, Burton MPM. Comparative utility of egg blastomere morphology and lipid biochemistry for prediction of hatching success in Atlantic cod, *Gadus morhua* L. *Aquaculture Research* 2006b; 37: 272-83.
- Pilet MF, Dousset X, Barre R, Novel G, Desmazeaud M, Piard JC. Evidence for 2 bacteriocins produced by *Carnobacterium piscicola* and *Carnobacterium divergens* isolated from fish and active against *Listeria monocytogenes*. *Journal of Food Protection* 1995; 58: 256-62.
- Pinhassi J, Zweifel UL, Hagstrom A. Dominant marine bacterioplankton species found among colony-forming bacteria. *Applied and Environmental Microbiology* 1997; 63: 3359-66.
- Planas M, Perez-Lorenzo M, Hjelm M, Gram L, Fiksdal IU, Bergh O, Pintado J. Probiotic effect *in vivo* of *Roseobacter* strain 27-4 against *Vibrio* (*Listonella*) *anguillarum* infections in turbot (*Scophthalmus maximus* L.) larvae. *Aquaculture* 2006; 255: 323-33.
- Plante S, Pernet F, Hache R, Ritchie R, Ji BJ, McIntosh D. Ontogenetic variations in lipid class and fatty acid composition of haddock larvae *Melanogrammus aeglefinus* in relation to changes in diet and microbial environment. *Aquaculture* 2007; 263: 107-21.
- Prol-Garcia MJ, Planas M, Pintado J. Different colonization and residence time of *Listonella anguillarum* and *Vibrio splendidus* in the rotifer *Brachionus plicatilis* determined by real-time PCR and DGGE. *Aquaculture* 2010; 302: 26-35.
- Pujalte MJ, Sitja-Bobadilla A, Macian MC, Alvarez-Pellitero P, Garay E. Occurrence and virulence of *Pseudoalteromonas* spp. in cultured gilthead sea bream (*Sparus aurata* L.) and European sea bass (*Dicentrarchus labrax* L.). Molecular and phenotypic characterisation of *P. undina* strain U58. *Aquaculture* 2007; 271: 47-53.
- Puvanendran V, Brown JA. Foraging, growth and survival of Atlantic cod larvae reared in different light intensities and photoperiods. *Aquaculture* 2002; 214: 131-51.
- Puvanendran V, Burt AL, Brown JA. Can Atlantic cod (*Gadus morhua*) larvae be weaned faster onto dry feed at higher temperatures? *Aquaculture* 2006; 255: 334-40.
- Puvanendran V, Calder-Crewe C, Brown JA. Vertebral deformity in cultured Atlantic cod larvae: ontogeny and effects on mortality. *Aquaculture Research* 2009; 40: 1653-60.
- Puvanendran V, Salies K, Laurel B, Brown JA. Size-dependent foraging of larval Atlantic cod (*Gadus morhua*). *Canadian Journal of Zoology-Revue Canadienne De Zoologie* 2004; 82: 1380-89.
- Qi ZZ, Dierckens K, Defoirdt T, Sorgeloos P, Boon N, Bao ZM, Bossier P. Effects of feeding regime and probionts on the diverting microbial communities in rotifer *Brachionus* culture. *Aquaculture International* 2009; 17: 303-15.
- Quan ZX, Xiao YP, Roh SW, Nam YD, Chang HW, Shin KS, Rhee SK, Park YH, Bae JW. *Joostella marina* gen. nov., sp nov., a novel member of the family Flavobacteriaceae isolated from the East Sea. *International Journal of Systematic and Evolutionary Microbiology* 2008; 58: 1388-92.

- Rahman M, Colque-Navarro P, Kuhn I, Huys G, Swings J, Mollby R. Identification and characterization of pathogenic *Aeromonas veronii* biovar *sobria* associated with epizootic ulcerative syndrome in fish in Bangladesh. *Applied and Environmental Microbiology* 2002; 68: 650-55.
- Rana KJ, (2007) Regional review on aquaculture development. 6. Western-European region - 2005. In: FAO Fisheries Circular No. 1017/6. Nations FaAOotU (ed). Rome, pp. 56.
- Reid HI, Treasurer JW, Adam B, Birkbeck TH. Analysis of bacterial populations in the gut of developing cod larvae and identification of *Vibrio logei*, *Vibrio anguillarum* and *Vibrio splendidus* as pathogens of cod larvae. *Aquaculture* 2009; 288: 36-43.
- Reitan KI, Rainuzzo JR, Oie G, Olsen Y. A review of the nutritional effects of algae in marine fish larvae. *Aquaculture* 1997; 155: 207-21.
- Remminghorst U, Rehm BHA. Bacterial alginates: from biosynthesis to applications. *Biotechnology Letters* 2006; 28: 1701-12.
- Rengpipat S, Tunyanun A, Fast AW, Piyatiratitivorakul S, Menasveta P. Enhanced growth and resistance to *Vibrio* challenge in pond-reared black tiger shrimp *Penaeus monodon* fed a *Bacillus* probiotic. *Diseases of Aquatic Organisms* 2003; 55: 169-73.
- Reyes-Becerril M, Salinas I, Cuesta A, Meseguer J, Tovar-Ramirez D, Ascencio-Valle F, Esteban MA. Oral delivery of live yeast *Debaryomyces hansenii* modulates the main innate immune parameters and the expression of immune-relevant genes in the gilthead seabream (*Sparus aurata* L.). *Fish & Shellfish Immunology* 2008; 25: 731-39.
- Ringo E. The effect of chromic oxide (CR₂O₃) on aerobic bacterial populations associated with the intestinal epithelial mucosa of Arctic charr, *Salvelinus alpinus* (L.). *Canadian Journal of Microbiology* 1993; 39: 1169-73.
- Ringo E. Does *Carnobacterium divergens* isolated from Atlantic salmon, *Salmo salar* L., colonize the gut of early developing turbot, *Scophthalmus maximus* L., larvae? *Aquaculture Research* 1999; 30: 229-32.
- Ringo E. The ability of carnobacteria isolated from fish intestine to inhibit growth of fish pathogenic bacteria: a screening study. *Aquaculture Research* 2008; 39: 171-80.
- Ringo E, Birkbeck TH. Intestinal microflora of fish larvae and fry. *Aquaculture Research* 1999; 30: 73-93.
- Ringo E, Birkbeck TH, Munro PD, Vadstein O, Hjelmeland K. The effect of early exposure to *Vibrio pelagius* on the aerobic bacterial flora of turbot, *Scophthalmus maximus* (L) larvae. *Journal of Applied Bacteriology* 1996; 81: 207-11.
- Ringo E, Myklebust R, Mayhew TM, Olsen RE. Bacterial translocation and pathogenesis in the digestive tract of larvae and fry. *Aquaculture* 2007; 268: 251-64.
- Ringo E, Sperstad S, Myklebust R, Refstie S, Krogdahl A. Characterisation of the microbiota associated with intestine of Atlantic cod (*Gadus morhua* L.) - The effect of fish meal, standard soybean meal and a bioprocessed soybean meal. *Aquaculture* 2006; 261: 829-41.
- Ringo E, Vadstein O. Colonization of *Vibrio pelagius* and *Aeromonas caviae* in early developing turbot (*Scophthalmus maximus* L.) larvae. *Journal of Applied Microbiology* 1998; 84: 227-33.
- Robertson PAW, O'Dowd C, Burrells C, Williams P, Austin B. Use of *Carnobacterium* sp as a probiotic for Atlantic salmon (*Salmo salar* L.) and rainbow trout (*Oncorhynchus mykiss*, Walbaum). *Aquaculture* 2000; 185: 235-43.
- Rocha RJ, Ribeiro L, Costa R, Dinis MT. Does the presence of microalgae influence fish larvae prey capture? *Aquaculture Research* 2008; 39: 362-69.

- Rollo A, Sulpizio R, Nardi M, Silvi S, Orpianesi C, Caggiano M, Cresci A, Carnevali O. Live microbial feed supplement in aquaculture for improvement of stress tolerance. *Fish Physiology and Biochemistry* 2006; 32: 167-77.
- Rombaut G, Suantika G, Boon N, Maertens S, Dhert P, Top E, Sorgeloos P, Verstraete W. Monitoring of the evolving diversity of the microbial community present in rotifer cultures. *Aquaculture* 2001; 198: 237-52.
- Rønnestad I, Thorsen A, Finn RN. Fish larval nutrition: a review of recent advances in the roles of amino acids. *Aquaculture* 1999; 177: 201-16.
- Rosenlund G, Halldorsson O. Cod juvenile production: Research and commercial developments. *Aquaculture* 2007; 268: 188-94.
- Roszak DB, Colwell RR. Survival strategies of bacteria in the natural environment. *Microbiological Reviews* 1987; 51: 365-79.
- Rust MB, Hardy RW, Stickney RR. A new method for force-feeding larval fish. *Aquaculture* 1993; 116: 341-52.
- Sakai M. Current research status of fish immunostimulants. *Aquaculture* 1999; 172: 63-92.
- Salinas I, Cuesta A, Esteban MA, Meseguer J. Dietary administration of *Lactobacillus delbrueckii* and *Bacillus subtilis*, single or combined, on gilthead seabream cellular innate immune responses. *Fish & Shellfish Immunology* 2005; 19: 67-77.
- Salvesen I, Oie G, Vadstein O. Surface disinfection of Atlantic halibut and turbot eggs with glutaraldehyde: Evaluation of concentrations and contact times. *Aquaculture International* 1997; 5: 249-58.
- Salvesen I, Reitan KI, Skjermo J, Oie G. Microbial environments in marine larviculture: Impacts of algal growth rates on the bacterial load in six microalgae. *Aquaculture International* 2000; 8: 275-87.
- Salvesen I, Skjermo J, Vadstein O. Growth of turbot (*Scophthalmus maximus* L.) during first feeding in relation to the proportion of r/K-strategists in the bacterial community of the rearing water. *Aquaculture* 1999; 175: 337-50.
- Salvesen I, Vadstein O. Surface disinfection of eggs from marine fish: evaluation of four chemicals. *Aquaculture International* 1995; 3: 155-71.
- Sandlund N, Bergh O. Screening and characterisation of potentially pathogenic bacteria associated with Atlantic cod *Gadus morhua* larvae: bath challenge trials using a multidish system. *Diseases of Aquatic Organisms* 2008; 81: 203-17.
- Schmidt TM, DeLong EF, Pace NR. Analysis of a marine picoplankton community by 16S ribosomal RNA gene cloning and sequencing. *Journal of Bacteriology* 1991; 173: 4371-78.
- Schroder MB, Villena AJ, Jorgensen TO. Ontogeny of lymphoid organs and immunoglobulin producing cells in Atlantic cod (*Gadus morhua* L.). *Developmental and Comparative Immunology* 1998; 22: 507-17.
- Schulze AD, Alabi AO, Tattersall-Sheldrake AR, Miller KM. Bacterial diversity in a marine hatchery: Balance between pathogenic and potentially probiotic bacterial strains. *Aquaculture* 2006; 256: 50-73.
- Schwenteit J, Gram L, Nielsen KF, Fridjonsson OH, Bornscheuer UT, Gisvkvov M, Gudmundsdottir BK. Quorum sensing in *Aeromonas salmonicida* subsp. *achromogenes* and the effect of the autoinducer synthase AsaI on bacterial virulence. *Veterinary Microbiology* 2010; doi:10.1016/j.vetmic.2010.07.020.
- Schwyn B, Neilands JB. Universal chemical assay for the detection and determination of siderophores. *Analytical Biochemistry* 1987; 160: 47-56.
- Seppola M, Johnsen H, Mennen S, Myrnes B, Tveiten H. Maternal transfer and transcriptional onset of immune genes during ontogenesis in Atlantic cod. *Developmental and Comparative Immunology* 2009; 33: 1205-11.

- Seppola M, Olsen RE, Sandaker E, Kanapathippillai P, Holzapfel W, Ringo E. Random amplification of polymorphic DNA (RAPD) typing of carnobacteria isolated from hindgut chamber and large intestine of Atlantic cod (*Gadus morhua* L.). *Systematic and Applied Microbiology* 2006; 29: 131-37.
- Serrano PH, (2005) Responsible use of antibiotics in aquaculture. In: FAO Fisheries Technical Paper No. 469. Rome: Food and Agricultural Organization of the United Nations, pp. 97.
- Shields RJ, Bell JG, Luizi FS, Gara B, Bromage NR, Sargent JR. Natural copepods are superior to enriched *Artemia* nauplii as feed for halibut larvae (*Hippoglossus hippoglossus*) in terms of survival, pigmentation and retinal morphology: Relation to dietary essential fatty acids. *Journal of Nutrition* 1999; 129: 1186-94.
- Skjermo J, Rokstad AM, Salvesen I, Skjåk-Bræk G, Vadstein O, (2002) Immunostimulation in marine larval rearing In: 2002 ICES Annual Science Conference and ICES Centenary Proceedings. Copenhagen, Denmark: ICES, pp. 1-6, <http://www.ices.dk/products/CMdocs/2002/R/R0602.PDF>.
- Skjermo J, Salvesen I, Oie G, Olsen Y, Vadstein O. Microbially matured water: A technique for selection of a non-opportunistic bacterial flora in water that may improve performance of marine larvae. *Aquaculture International* 1997; 5: 13-28.
- Skjermo J, Storseth TR, Hansen K, Handa A, Oie G. Evaluation of β -(1 $\rightarrow$ 3, 1 $\rightarrow$ 6)-glucans and high-M alginate used as immunostimulatory dietary supplement during first feeding and weaning of Atlantic cod (*Gadus morhua* L.). *Aquaculture* 2006; 261: 1088-101.
- Skjermo J, Vadstein O. Characterization of the bacterial flora of mass cultivated *Brachionus plicatilis*. *Hydrobiologia* 1993; 255: 185-91.
- Skjermo J, Vadstein O. Techniques for microbial control in the intensive rearing of marine larvae. *Aquaculture* 1999; 177: 333-43.
- Smacchi E, Gobetti M, Lanciotti R, Fox PF. Purification and characterization of an extracellular proline iminopeptidase from *Arthrobacter nicotianae* 9458. *FEMS Microbiology Letters* 1999; 178: 191-97.
- Sommerset I, Krossoy B, Biering E, Frost P. Vaccines for fish in aquaculture. *Expert Review of Vaccines* 2005; 4: 89-101.
- Spanggaard B, Huber I, Nielsen J, Nielsen T, Appel KF, Gram L. The microflora of rainbow trout intestine: a comparison of traditional and molecular identification. *Aquaculture* 2000; 182: 1-15.
- Spanggaard B, Huber I, Nielsen J, Sick EB, Pipper CB, Martinussen T, Slierendrecht WJ, Gram L. The probiotic potential against vibriosis of the indigenous microflora of rainbow trout. *Environmental Microbiology* 2001; 3: 755-65.
- Spotte SH. *Seawater Aquariums: The Captive Environment*. NY, USA: Wiley-Interscience, John Wiley and Sons; 1979: 413.
- Srivastava A, Hamre K, Stoss J, Chakrabarti R, Tonheim SK. Protein content and amino acid composition of the live feed rotifer (*Brachionus plicatilis*): With emphasis on the water soluble fraction. *Aquaculture* 2006; 254: 534-43.
- Stanbridge LH, Board RG. A modification of the *Pseudomonas* selective medium, CFC, that allows differentiation between meat pseudomonads and Enterobacteriaceae. *Letters in Applied Microbiology* 1994; 18: 327-28.
- Steinarsson A. Hatchery production of cod. In: Björnsson B, Gunnarsson VI, (eds). *Cod Farming in Iceland*, Report nr.111. Marine Research Institute, Reykjavik, 2004: pp. 41-86 (in Icelandic, with English abstract).
- Steinarsson A, Björnsson B. The effects of temperature and size on growth and mortality of cod. *Journal of Fish Biology* 1999; 55: 100-09.

- Strom E, Olafsen J. The indigenous microflora of wild-captured juvenile cod in net-pen rearing. In: L  sel R, (ed). Microbiology of Poecilotherms. Amsterdam: Elsevier, 1990: 181-85.
- Strom E, Ringo E. Changes in bacterial flora in developing cod, *Gadus morhua* L., larvae after inoculation of *Lactobacillus plantarum* in the water. In: B Walther HF, (ed). Physiological and Biochemical Aspects of Fish Larval Development. Bergen: University of Bergen, 1993: 226-28.
- Suzer C, Coban D, Kamaci HO, Saka S, Firat K, Otgucuoglu O, Kucuksari H. *Lactobacillus* spp. bacteria as probiotics in gilthead sea bream (*Sparus aurata*, L.) larvae: Effects on growth performance and digestive enzyme activities. Aquaculture 2008; 280: 140-45.
- Sveinsdottir H, Steinarsson A, Gudmundsdottir A. Differential protein expression in early Atlantic cod larvae (*Gadus morhua*) in response to treatment with probiotic bacteria. Comparative Biochemistry and Physiology Part D-Genomics & Proteomics 2009; 4: 249-54.
- Sveinsdottir H, Thorarensen H, Gudmundsdottir A. Involvement of trypsin and chymotrypsin activities in Atlantic cod (*Gadus morhua*) embryogenesis. Aquaculture 2006; 260: 307-14.
- Swain SM, Singh C, Arul V. Inhibitory activity of probiotics *Streptococcus phocae* PI80 and *Enterococcus faecium* MC13 against vibriosis in shrimp *Penaeus monodon*. World Journal of Microbiology & Biotechnology 2009; 25: 697-703.
- Tait K, Williamson H, Atkinson S, Williams P, Camara M, Joint I. Turnover of quorum sensing signal molecules modulates cross-kingdom signalling. Environmental Microbiology 2009; 11: 1792-802.
- Taoka Y, Maeda H, Jo JY, Kim SM, Park SI, Yoshikawa T, Sakata T. Use of live and dead probiotic cells in tilapia *Oreochromis niloticus*. Fisheries Science 2006; 72: 755-66.
- Thomson R, Macpherson HL, Riaza A, Birkbeck TH. *Vibrio splendidus* biotype 1 as a cause of mortalities in hatchery-reared larval turbot, *Scophthalmus maximus* (L.). Journal of Applied Microbiology 2005; 99: 243-50.
- Timmons MB, Ebeling JM, Wheaton FW, Summerfelt ST, Vinci BJ. *Recirculating Aquaculture Systems, 2nd edition*. NY, USA: Cayuga Aqua Ventures; 2002: 769.
- Tinh NTN, Dierckens K, Sorgeloos P, Bossier P. A review of the functionality of probiotics in the larviculture food chain. Marine Biotechnology 2008a; 10: 1-12.
- Tinh NTN, Yen VHN, Dierckens K, Sorgeloos P, Bossier P. An acyl homoserine lactone-degrading microbial community improves the survival of first-feeding turbot larvae (*Scophthalmus maximus* L.). Aquaculture 2008b; 285: 56-62.
- Tocher DR, Mourente G, Van der Eecken A, Evjemo JO, Diaz E, Wille M, Bell JG, Olsen Y. Comparative study of antioxidant defence mechanisms in marine fish fed variable levels of oxidised oil and vitamin E. Aquaculture International 2003; 11: 195-216.
- Tomasso JR. Toxicity of nitrogenous wastes to aquaculture animals. Reviews in Fisheries Science 1994; 2: 291-314.
- Treasurer JW, Cochrane E, Grant A. Surface disinfection of cod *Gadus morhua* and haddock *Melanogrammus aeglefinus* eggs with bronopol. Aquaculture 2005; 250: 27-35.
- van der Meeren T, Olsen RE, Hamre K, Fyhn HJ. Biochemical composition of copepods for evaluation of feed quality in production of juvenile marine fish. Aquaculture 2008; 274: 375-97.
- Vendrell D, Balcazar JL, de Blas I, Ruiz-Zarzuela I, Girones O, Muzquiz JL. Protection of rainbow trout (*Oncorhynchus mykiss*) from lactococcosis by probiotic bacteria. Comparative Immunology Microbiology and Infectious Diseases 2008; 31: 337-45.

- Verdonck L, Swings J, Kersters K, Dehasque M, Sorgeloos P, Leger P. Variability of the microbial environment of rotifer *Brachionus plicatilis* and *Artemia* production systems. *Journal of the World Aquaculture Society* 1994; 25: 55-59.
- Verner-Jeffreys DW, Shields RJ, Bricknell IR, Birkbeck TH. Effects of different water treatment methods and antibiotic addition on larval survival and gut microflora development in Atlantic halibut (*Hippoglossus hippoglossus* L.) yolk-sac larvae. *Aquaculture* 2004; 232: 129-43.
- Verschuere L, Rombaut G, Sorgeloos P, Verstraete W. Probiotic bacteria as biological control agents in aquaculture. *Microbiology and Molecular Biology Reviews* 2000; 64: 655-71.
- Vijayan KK, Singh ISB, Jayaprakash NS, Alavandi SV, Pai SS, Preetha R, Rajan JJS, Santiago TC. A brackishwater isolate of *Pseudomonas* PS-102, a potential antagonistic bacterium against pathogenic vibrios in penaeid and non-penaeid rearing systems. *Aquaculture* 2006; 251: 192-200.
- Villamil L, Figueras A, Planas M, Novoa B. Control of *Vibrio alginolyticus* in *Artemia* culture by treatment with bacterial probiotics. *Aquaculture* 2003; 219: 43-56.
- Vine NG, Leukes WD, Kaiser H. In vitro growth characteristics of five candidate aquaculture probiotics and two fish pathogens grown in fish intestinal mucus. *FEMS Microbiology Letters* 2004; 231: 145-52.
- Vollset KW, Fiksen O, Folkvord A. Vertical distribution of larval cod (*Gadus morhua*) in experimental temperature gradients. *Journal of Experimental Marine Biology and Ecology* 2009; 379: 16-22.
- Wang YB, Tian ZQ, Yao JT, Li WF. Effect of probiotics, *Enterococcus faecium*, on tilapia (*Oreochromis niloticus*) growth performance and immune response. *Aquaculture* 2008; 277: 203-07.
- Waters CM, Bassler BL. Quorum sensing: Cell-to-cell communication in bacteria. *Annual Review of Cell and Developmental Biology* 2005; 21: 319-46.
- Wold PA, Hoehne-Reitan K, Cahu CL, Infante JZ, Rainuzzo J, Kjorsvik E. Phospholipids vs. neutral lipids: Effects on digestive enzymes in Atlantic cod (*Gadus morhua*) larvae. *Aquaculture* 2007; 272: 502-13.
- Wold PA, Hoehne-Reitan K, Cahu CL, Infante JZ, Rainuzzo J, Kjorsvik E. Comparison of dietary phospholipids and neutral lipids: effects on gut, liver and pancreas histology in Atlantic cod (*Gadus morhua* L.) larvae. *Aquaculture Nutrition* 2009; 15: 73-84.
- Wold PA, Hoehne-Reitan K, Rainuzzo J, Kjorsvik E. Allometric growth and functional development of the gut in developing cod *Gadus morhua* L. larvae. *Journal of Fish Biology* 2008; 72: 1637-58.
- Yoon JH, Lee CH, Kang SJ, Oh TK. *Psychrobacter celer* sp nov., isolated from sea water of the South Sea in Korea. *International Journal of Systematic and Evolutionary Microbiology* 2005; 55: 1885-90.
- Zhang LH. Quorum quenching and proactive host defense. *Trends in Plant Science* 2003; 8: 238-44.
- Ziaei-Nejad S, Rezaei MH, Takami GA, Lovett DL, Mirvaghefi AR, Shakouri M. The effect of *Bacillus* spp. bacteria used as probiotics on digestive enzyme activity, survival and growth in the Indian white shrimp *Fenneropenaeus indicus*. *Aquaculture* 2006; 252: 516-24.

8 ORIGINAL PAPERS I-V

PAPER I

Isolation of putative probionts from cod rearing environment

Lauzon, H.L., Gudmundsdottir, S., Pedersen, M.H.,
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Isolation of putative probionts from cod rearing environment

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Abstract

Survival problems are encountered at early stages of intensive fish rearing and antibiotics are widely used to remedy the situation. Probiotics may provide a potential alternative method to protect larvae from opportunistic and pathogenic bacteria and promote a balanced environment. This study was designed to search for new probiotics to target this critical period in cod rearing. Potential probionts were selected from the natural microbiota of cod aquacultural environment. The selection was based on several criteria: pathogen inhibition potential, growth characteristics, strain identification, metabolite production and adhesion to fish cell lines. Our study demonstrated that 14% of screened bacteria ($n = 188$) had antagonistic properties towards fish pathogens. The majority of these isolates were Gram-positive (81%), belonging to Firmicutes (69.2%) and Actinobacteria (11.5%) phyla based on 16S rRNA gene sequencing. Only 6 (3.2%) of 188 isolates could inhibit all three pathogens tested: *Vibrio anguillarum*, *Aeromonas salmonicida* subsp. *achromogenes* and *Vibrio salmonicida*. Differences observed in activity intensity and spectrum among inhibitory isolates emphasise the need to develop probiotic mixtures for efficient prophylactic methods. Comparison of growth behaviour of inhibitory isolates and pathogens at cod rearing temperatures, metabolite production and adhesion capacity were considered for final probiont selection. Four promising isolates that could be used as a mixed supplement to rearing water were identified as putative probiotic bacteria. This study emphasises the importance and potential of lactic acid bacteria in aquaculture.

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Keywords: Cod aquaculture; Probiotic; Fish pathogens; Antagonism; Growth characteristics; Adhesion

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1. Introduction

Proliferation of opportunistic and pathogenic microorganisms in intensive rearing systems is known to cause poor larval growth and high mortality rates (Munro et al., 1995). Antibiotics have been used to prevent and control bacterial

PAPER II

Effects of bacterial treatment at early stages of Atlantic cod (*Gadus morhua* L.) on larval survival and development

Lauzon, H.L., Gudmundsdottir, S., Steinarsson, A.,
Oddgeirsson, M., Petursdottir, S.K., Reynisson, E.,
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ORIGINAL ARTICLE

Effects of bacterial treatment at early stages of Atlantic cod (*Gadus morhua* L.) on larval survival and development

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Keywords

cod larviculture, hypersalinity tolerance, larval development, probiotic, survival.

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Abstract

Aims: To assess the effects of bacterial treatment at the earliest stages of cod rearing on the microbial load, larval development and performance, testing three bacterial strains (*Carnobacterium divergens* V41, *Arthrobacter* sp. and *Enterococcus* sp.) *in vivo* that were previously shown to have inhibitory potential towards fish pathogens *in vitro*.

Methods and Results: A bacterial mixture was added eight times to the rearing water from the pre-hatch to the mid-larval stage (a 38-day period). Microbiological analysis of ova, larvae and rearing water was performed regularly. Larval performance and development were evaluated by survival rate, hypersalinity tolerance and physiological measurements. Different larval survival rates were observed within and between treatments, and possibly explained by variations in larval microflora and established probionts. Larvae from one silo, which had been bathed in the bacterial suspension, showed the highest survival rate (42.1%), lowest *Vibrio* levels, and were significantly heavier (19.3%) and more stress tolerant than control larvae ($P < 0.01$). This coincided with the intestinal establishment of two of the tested bacteria.

Conclusions: *Arthrobacter* and *Enterococcus* strains added regularly to the rearing water from the postfertilized egg stage can become established in larval gastrointestinal tract. The *Enterococcus* strain was associated with increased larval growth, performance and microflora control, indicating its probiotic nature.

Significance and Impact of the Study: Regular application of autochthonous probionts may promote larval welfare, development and stress tolerance at early stages, hence increasing production yield in intensive cod larviculture.

Introduction

New techniques need to be developed to increase the production yield in intensive larviculture of marine species. Use of antibiotics should be minimized to avoid the spread of antibiotic resistance genes in pathogenic bacteria and environmental contamination. Probiotics are a promising prophylactic measure, where live micro-organisms are administered in adequate amounts to confer a

health benefit on the host. A probiotic should have a proven safety and efficacy in the host (WHO website 10 April 2008; http://www.who.int/foodsafety/fs_management/en/probiotic_guidelines.pdf). In aquaculture, probiotic treatments through rearing water may be just as relevant as oral treatments (Gatesoupe 1999). Indeed, Verschuere *et al.* (2000) proposed a new definition, applicable to aquatic environments, as a live microbial adjunct, which benefits the host by modifying the host-associated

PAPER III

Microbiota of Atlantic cod (*Gadus morhua* L.) rearing systems at pre- and posthatch stages and the effect of different treatments

Lauzon, H.L., Gudmundsdottir, S., Petursdottir, S.K., Reynisson,
E., Steinarsson, A., Oddgeirsson, M.,
Bjornsdottir, R., Gudmundsdottir, B.K.

ORIGINAL ARTICLE

Microbiota of Atlantic cod (*Gadus morhua* L.) rearing systems at pre- and posthatch stages and the effect of different treatments

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Keywords

cod hatchery, haemolysin, microbiota, probiotic, quorum sensing, siderophore.

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Abstract

Aims: To study the effect of ova disinfection, antibiotic and microbial treatments on the dominant cultivable cod rearing microbiota at pre- and posthatch stages, determining some virulence-related phenotypic traits among bacterial isolates and their relation to larval survival.

Methods and Results: Sampling of rearing systems (rearing water, ova, larvae, feeds and supplement) for analysis of cultivable microbiota took place at early stages in 2004 and 2005. Cultivation, phenotypic and genotypic (16S rRNA gene) analyses were performed. The production of putative virulence factors (PVFs), including haemolysin, siderophores and quorum-sensing signals, by bacterial isolates was investigated and related to larval survival. The study was performed during two spawning seasons, evaluating current hatchery practices (ova disinfection and antibiotic treatment of unhealthy larvae) and specific putative probiotics applied to ova and larvae or rotifers. A diversified microbiota (75 operational taxonomic units, OTUs) was observed in cod rearing systems influenced by the feeds and treatments, with prevailing γ -Proteobacteria prior to hatching towards a multiphyla microbiota posthatch. Phenotypic tests demonstrated the heterogeneity within some OTUs. Multivariate analysis of survival data in larval silos and the corresponding larval microbiota was used to divide the genotypic groups into beneficial/harmless and detrimental/opportunistic clusters. PVFs were common among the proposed detrimental/opportunistic OTUs.

Conclusions: The results clearly demonstrate the influence of exogenous feeding and treatments on larval gastrointestinal microbiota and the role of bacteria in larval survival.

Significance and Impact of the Study: Increased understanding of the microbiota in rearing systems may contribute to successful implementation of microbial management in cod aquaculture.

Introduction

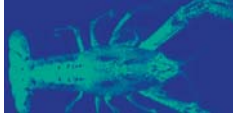
High mortality rate at early larval stage in intensive cod (*Gadus morhua* L.) hatcheries is common, but the reasons are not fully understood. Several factors may play a role,

such as suboptimal rearing conditions, nutritional status and immature immune system of larvae as well as detrimental or pathogenic micro-organisms. Apart from the invasion of recognized pathogens, poor larval viability may be caused by opportunistic bacteria that become

PAPER IV

Application of prospective probionts at early stages of Atlantic cod (*Gadus morhua* L.) rearing

Lauzon, H.L., Magnadottir, B., Gudmundsdottir, B.K.,
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Application of prospective probionts at early stages of Atlantic cod (*Gadus morhua* L.) rearing

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Abstract

This work aimed at validating the use of two prospective probionts (*Arthrobacter* sp. and *Enterococcus* sp.) at early stages of cod (*Gadus morhua* L.) rearing. Ova at late post-fertilized stage and larvae during their first 4 weeks of life were bathed with both probionts, isolated previously from the cod-rearing environment. This treatment was compared with groups fed rotifers supplemented with a commercial probiotic (Remus[®]) and those untreated. Microbiological analyses (total viable counts, presumptive *Vibrio* and lactic acid bacteria) were performed in rearing systems and larval survival, growth and development were assessed. Larval development was evaluated by proteolytic activity of larval lysates and immunological analysis of important proteins: apolipoprotein A-I, haemoglobin, C-reactive protein, C3 and cod serum proteins. Bacterial bathing led to a significantly higher larval weight, length and culturable microbial load in larval gastrointestinal (GI) tract when compared with the control and Remus groups. Development occurred earlier in bathed larvae. However, their survival was negatively affected compared with the control group, but was significantly higher than for the Remus group. The non-pathogenicity of both probionts was demonstrated by intraperitoneal injection of 13 g cod juveniles. The results suggest that *Arthrobacter* and *Enterococcus* probionts affected the larval GI microbiota and contributed to growth, development and digestion, either directly or indirectly.

Keywords: cod larviculture, probiotic, larval development, microbiota, immunological proteins

Introduction

Atlantic cod (*Gadus morhua* L.) is a new species in aquaculture, but its low survival rate and suboptimal growth affect the cost effectiveness of its production. The demand for environment-friendly and sustainable aquaculture has led to increasing research on probiotics for aquatic animals. Verschuere, Rombaut, Sorgeloos and Verstraete (2000) introduced a new definition relating to the application of probiotics in the aquatic environment: a live microbial adjunct, which benefits the host by modifying the host-associated or ambient microbial community, by enhancing the host response towards disease, by ensuring improved use of feed or enhancing its nutritional value or by improving the quality of its ambient environment. This suggests that two vectors can be used for probiont transmission to larvae, either via live feed or the rearing water as proposed by Gatesoupe (1999).

Several probionts tested in fish aquaculture have been selected based on their effects in humans (Nikoskelainen, Ouwehand, Salminen & Bylund 2001; Panigrahi, Kiron, Kobayashi, Puangkaew, Satoh & Sugita 2004; Balcazar, De Blas, Ruiz-Zarzuela, Vendrell, Calvo, Marquez, Girones & Muzquiz 2007) and livestock (Gatesoupe 2002; El-Haroun, Goda & Chowdhury 2006; Suzer, Coban, Kamaci, Saka, Firat, Otgucuoglu & Kucuksari 2008). These solutions may prove safe for human consumption but their efficiency in marine environment, as for coldwater fish, may be expected to be poor due to different requirements of non-marine, mesophilic microbes. Other researchers have focused their selection on autochthonous bacteria, i.e. those originating from the respective aquatic

PAPER V

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Impact of probiotic intervention on microbial load and performance of Atlantic cod (*Gadus morhua* L.) juveniles

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ABSTRACT

An intervention study was conducted at early juvenile stage of Atlantic cod (*Gadus morhua* L.) rearing to confirm the properties of two prospective probiotics, *Arthrobacter* sp. and *Enterococcus* sp. isolated from a cod hatchery. Juveniles (10 g) were fed expanded dry feed for 28 or 55 days, either untreated or treated with the probiotic bacteria in mixture or singly (10^{7-9} CFU g⁻¹ feed). Viability of probiotics supplemented to the feed was evaluated during a two-month storage at −20, 4 and 15 °C and sensory evaluation of the probiotic feed performed at the end of storage. Juvenile growth, survival, feed conversion ratio, and microbial load of rearing water, juvenile gills and gut were examined. Microbiological stability of the probiotics added to the feed was demonstrated at all temperatures, while sensory evaluation detected faint oxidative off-odours in feed stored frozen. Juveniles fed the *Enterococcus*-probiotic feed grew significantly faster and had significantly lower gut *Vibrio* counts than control juveniles ($p < 0.05$). Overall, the results suggest that both probiotics, especially the *Enterococcus* strain, modified the gut microbiota and contributed to enhanced juvenile growth and survival.

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1. Introduction

Research on the effect of probiotics administered to fish juveniles via feeding was initiated more than a decade ago and aimed mainly at assessing their prophylactic effect. Lactic acid bacteria (LAB) have been administered to various fish species and increased survival demonstrated in subsequent pathogen challenge studies in cod (Gildberg and Mikkelsen, 1998), trout (Irianto and Austin, 2002; Nikoskelainen et al., 2001; Vendrell et al., 2008), tilapia (Aly et al., 2008) and eel (Chang and Liu, 2002). Pathogen resistance has been observed in studies testing both Gram-positive and Gram-negative bacterial species (Brunt and Austin, 2005; Irianto and Austin, 2002) along with increased growth (Ghosh et al., 2008; Rengpipat et al., 2003).

Immune enhancement in fish has been reported in trout (Brunt and Austin, 2005; Nikoskelainen et al., 2003; Panigrahi et al., 2004; Panigrahi et al., 2007), seabream (Salinas et al., 2005) and tilapia (Wang et al., 2008) given LAB-supplemented diets. Effects of LAB on

gut microbiota have been reported in sheat fish (Bogut et al., 2000), eel (Chang and Liu, 2002), trout (Panigrahi et al., 2004) and ornamental fish species (Ghosh et al., 2008). Recently, a commercial probiotic product, Biogen®, was shown to increase growth performance in tilapia (El-Haroun et al., 2006).

Many probiotics tested in fish have been selected due to their efficiency in humans and livestock (Azad and Ai-Marzouk, 2008). Currently, some commercial probiotic products targeting warmwater host species are available worldwide, while little information is provided on their efficiency for coldwater fish such as cod. The first zootechnical aquaculture feed additive, Bactocell® (*Pediococcus acidilactici*, Lallemand Animal Nutrition, France) was recently authorised in Europe for salmonids and shrimp due to its safety and efficacy in beneficially enhancing production (http://www.lallemand.com/Home/eng/PDFs/LAN09-PR09-Bactocell_aqua_EN.pdf; retrieved 11.02.10). It has been used in feed for fattening pigs and chickens since 2005.

Recently, potential probiotics were selected from the natural microbiota of cod larviculture based on several *in vitro* tests, including their growth characteristics and inhibition of fish pathogens (Lauzon et al., 2008). Two potential bacterial strains, supplied contemporaneously to the rearing water at early cod larval stages, were reisolated in larvae at the end of the experiment resulting in higher survival, growth and stress tolerance as well as microbiota control (Lauzon et al., 2010b). The aim of this study was to validate the use of both probiotics at early cod juvenile stage through bacteria-supplemented

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