The bacterial community during early production stages of intensively reared halibut (*Hippoglossus hippoglossus* L.)

Rannveig Björnsdóttir

Thesis for the degree of Philosophiae Doctor (Ph.D.)
University of Iceland
Faculty of Medicine
School of Helath Sciences

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Margt er valt í veröldinni,
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Dissertation for the Degree of Ph.D. in Biomedical Sciences

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ÁGRIP

Mikil og skyndileg afföll verða á fyrstu stigum eldis sjávartegunda fiska og gefa rannsóknir vísbendingar um að bakteríuvöxtur hafi víðtækari áhrif á vöxt og afkomu lirfa en áður er þekkt. Bakteríur nærast á lífrænum efnum og úrgangi í eldiskerfunum og geta óæskilegar tegundir því náð miklum fjölda á skömmum tíma. Áhrif einstakra tegunda eru þó lítt þekkt en mikilvægt er að eldisumhverfi lirfanna sé stöðugt og sem minnstar breytingar verði á þessum viðkvæmu stigum eldisins.

Bakteríuflóra hrogna og lirfa lúðu (*Hippoglossus hippoglossus* L.), eldisumhverfis þeirra og fæðudýra var kortlögð með ræktun á næringarætum svo og með sameindalíffræðilegum aðferðum (denaturing gradient gel electrophoresis, DGGE), sem ekki krefjast ræktunar bakteríanna. Ennfremur voru könnuð áhrif mismunandi meðhöndlunar á samsetningu bakteríuflóru svo og gæði hrogna og lirfa. Þetta var gert með samanburði á áhrifum skyggingar eldisvökva í kerjum ýmist með þörungum eða leir, meðhöndlun fóðurdýra lirfa með vatnsrofnum fiskipróteinum og meðhöndlun hrogna og fóðurdýra lirfa með blöndu þriggja baktería (*Pseudoalteromonas elyakovii*, *Vibrio* spp. og *V. splendidus*), sem einangraðar voru úr meltingarvegi lirfa í kerjum með góða afkomu.

Bakteríuflóra kviðpokalirfa reyndist fjölbreyttari en áður hefur verið sýnt fram á og samband fannst á milli kjaftgalla og fjölda ræktanlegra baktería í lirfum. *Vibrio* tegundir voru ríkjandi í meltingarvegi lirfa eftir að frumfóðrun hófst. Einnig voru α-Proteobacteria einangraðar sem hluti af ríkjandi flóru lirfa en þessi hópur hefur ekki áður verið staðfestur í lúðu. Bakteríuflóra í lifandi fóðurdýrum lirfa reyndist aðeins endurspeglast að hluta til í meltingarvegi lirfa eftir fyrstu vikuna í fóðrun. Einungis lítill hluti tegunda í ákaflega fjölbreyttri bakteríuflóru lirfa og fóðurdýra þeirra reyndist vera ræktanlegur en vísbendingar eru um að ríkjandi hluti flórunnar geti að stórum hluta verið ræktanlegur.

Notkun leirs í stað þörunga við nauðsynlega skyggingu eldisumhverfis lirfa hafði ekki áhrif á vöxt, gæði eða afkomu lirfa í startfóðrun en veruleg fækkun varð á fjölda baktería í eldisumhverfi lirfa. Meðhöndlun með stofnum sem einangraðir voru úr ríkjandi flóru í meltingarvegi lirfa úr kerum með góða afkomu hafði ekki áhrif á lifun eða gæði hrogna

en fóðrun með bakteríumeðhöndluðum fæðudýrum leiddi til aukinnar lifunar og marktæk aukning var í vexti lirfanna í annarri af tveimur tilraunum sem framkvæmdar voru.

Meðhöndlun fæðudýra með vatnsrofnum fiskipróteinum örvaði ósérhæft ónæmi lirfa, þar sem framleiðsla lysósíms var aukin og meiri dreifing var á C3 þætti komplement kerfisins í vefjum. Meðhöndlunin hafði hins vegar hvorki áhrif á bakteríuflóru né lifun lirfa. Meðfætt IgM greindist í lirfum við upphaf startfóðrunar rúmum mánuði eftir klak en meðfætt IgM hefur ekki áður verið greint í lúðulirfum.

Lykilorð: lúðueldi; bakteríuflóra; PCR-DGGE; bætibakteríur; ónæmisörvun

ABSTRACT

High bacterial numbers and the establishment of an unfavourable bacterial community has been identified as possible causes of the high mortalities commonly observed during early life stages of intensively reared Atlantic halibut (*Hippoglossus hippoglossus* L.). The impact of particular bacterial species is, however, poorly defined and still remains disputable.

Highly variable larval survival and overall success were observed and analysis of the bacterial community revealed a high variation in the cultivable part as well as the bacterial community structure as analysed by denaturing gradient gel electrophoresis (DGGE) of PCR amplified 16S ribosomal DNA fragments. The effects of different treatments on the bacterial community and the quality of eggs and larvae were analysed. The treatments involved shading of the culture environment of larvae using either marine algae or inorganic clay, bioencapsulation of the live prey using a fish protein hydrolysate and treatment of eggs and bioencapsulation of the live prey using a mixture of three bacteria, *Pseudoalteromonas elyakovii*, *Vibrio* spp. and *V. splendidus* that were selected from the dominating bacterial community of overall successful larvae.

Analysis of the bacterial community of unfed yolk sac larvae revealed a higher diversity than previously reported, with a positive relationship commonly observed between jaw deformation and the numbers of cultivable bacteria in unfed yolk sac larvae. *Vibrio* spp. dominated the bacterial community of larvae after the onset of exogenous feeding and α-Proteobacteria, not previously reported in halibut, were observed as a part of the intestinal microbiota. The bacterial community structure of the live prey was only partly reflected in larvae after one week of feeding, and the diverse bacterial community was only partly reflected in the cultivable part which may, however, reflect the dominating bacterial microbiota of larvae and their live prey.

Higher bacterial numbers were observed in the tank water of larvae with environmental shading provided by marine algae as compared with inorganic clay. Manipulation of the bacterial community using isolates dominating the cultivable gut community of overall

successful larvae did not affect egg survival, while bioencapsulation of the live prey resulted in enhanced larval survival, and improved larval growth was observed in one of the two experiments that were carried out. Bioencapsulation of the live prey using a fish protein hydrolysate resulted in stimulation of an innate immune response, with enhanced production of lysozyme, and more widespread distribution of complement factor C3 in larval tissues observed. Treatment was, however, neither found to affect the bacterial community associated with surface sterilized larvae nor larval survival and development. Low concentrations of IgM detected in larvae at the onset of feeding indicated maternal origin, not previously reported in halibut larvae.

Keywords: Atlantic halibut; bacterial community; PCR-DGGE; bacterial treatment; immunostimulation

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DECLARATION OF CONTRIBUTION

I was responsible for conception, strategic planning and management of the research. I furthermore supervised and was responsible for the work carried out by my collaborators at Matís ohf. in Akureyri and MSc students at the University of Akureyri, who participated in some of the work in their research connected studies. I participated in the experimental planning, experimental set-up and implementation, selection of isolates for probiotic treatments, treatments and sampling at the experimental fish farm, as well as in the microbiological and immunological analysis. The studies were partly funded by various research funds in Iceland and I wrote or supervised the writing of application proposals for grants. I also gathered reference material and performed overall data analyses. I wrote the first drafts of manuscripts I-III and participated in the writing of manuscript IV through supervision of my MSc student. I and my supervisor were responsible for the writing of the four manuscripts. All authors were involved in editing and the final approval of the manuscripts.

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LIST OF ABBREVIATIONS

ARA	Arachidonic Acid
BSA	Bovine Serum Albumin
C3	Complement factor 3
CFU	Colony Forming Units
dpf	days post fertilization
dph	days post hatching
dpff	days post onset of first feeding
DGGE	Denaturing Gradient Gel Electrophoresis
DHA	DocosaHexaenoic Acid
DNA	DeoxyriboNucleic Acid
rDNA	ribosomal DeoxyriboNucleic Acid
ELISA	Enzyme Linked ImmunoSorbent Assay
EPA	EicosaPentaenoic Acid
FISH	Fluorescence *in situ* Hybridization
High-M	High-mannuronic acid
HRP	HorseRadish Peroxidase
HUFA	Highly Unsaturated Fatty Acids
IgG	Immunoglobulin G
IgM	Immunoglobulin M
MA	Marine Agar
mAb	monoclonal Antibodies
PAMPs	Pathogen-Associated Molecular Patterns
PBS	Phosphate Buffered Saline
PBSA	Phosphate Buffered bovine Serum Albumin
pers. comm.	personal communication
ppm	part per million

PS	Peptone-Seawater
sw	seawater
T_3	Triiodothyronine
T_4	Thyroxine
TCBS	Thiosulphate Citrate Bile Salt Sucrose agar
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
PCR	Polymerase Chain Reaction
RNA	RiboNucleic Acid
rRNA	ribosomal RiboNucleic Acid
mRNA	messenger RiboNucleic Acid
TGGE	Temperature Gradient Gel Electrophoresis
TLRs	Toll-Like Receptors
UV	UltraViolet

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II. Using inorganic clay as compared to marine microalgae for environmental shading during first feeding of Atlantic halibut *Hippoglossus hippoglossus* larvae: Effects on the cultivable bacterial community and larval survival. (Aquaculture Research *Submitted May 2009*).

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III. Selection of bacteria and the effects of bacterial treatment of Atlantic halibut (*Hippoglossus hippoglossus* L.) eggs and larvae. (Aquaculture *Accepted for publication February 2010*).

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IV. Analysis of effects induced by a pollock protein hydrolysate on early development, innate immunity and the bacterial community structure of first feeding of Atlantic halibut (*Hippoglossus hippoglossus* L.) larvae. Fish and Shellfish Immunology 27 (2009) 595-602.

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1 INTRODUCTION

1.1 Atlantic halibut in commercial aquaculture

Most of the main fishing areas of the world have reached their maximum sustainable yield, with a general worldwide decline in the wild fisheries of marketable species parallel to increasing demands for proteins to supply the continually growing human population (FAO, 2006; 2009; Naylor and Burke, 2005). Hence, aquaculture has become critical and is a growing industry worldwide, with a continual increase in production quantities foreseen (FAO, 2009). Atlantic halibut (*Hippoglossus hippoglossus* L.) is a cold-water marine species native to the North Atlantic and, due to its highly prized white flesh containing rich amounts of healthy and beneficial fatty acids, the species is considered a valuable candidate for commercial farming (Ginsberg and Toal, 2009; Naylor and Burke, 2005). High mortality during early stages is, however, a well recognized problem and the production of larvae has been the main obstacle for the development of Atlantic halibut farming (Jensen *et al.*, 2004a; Kvåle *et al.*, 2007; Olsen *et al.*, 1999b).

1.2 Intensive production of Atlantic halibut larvae

1.2.1 Production methods and success

Maintaining optimum environmental conditions with proper nutrition and feeding together with general husbandry practices is of critical importance for successful performance of cultured fish. The appropriate light conditions, temperature, salinity and various water-quality factors furthermore affect normal development and hence, the overall production success (Howell and Baynes, 2004; Olsen *et al.*, 2004; Stoss *et al.*, 2004). A mean survival of up to 0-20% is commonly observed from fertilization of eggs through larval metamorphosis during intensive production of halibut larvae (*pers. comm.* Heiddís Smáradóttir, Fiskey Ltd.).

Seasonally independent egg and fry production has been achieved for halibut by photoperiod and temperature manipulation of broodstock, with groups spawning at advanced and delayed periods, however, without differences in observable egg quality (Pavlov *et al*., 2004; *pers. comm.* Heiddís Smáradóttir, Fiskey Ltd.). The relatively large

halibut eggs (Falk-Petersen, 2005) are obtained by stripping, with approximately 60% success of fertilization obtained with large scale production (*pers. comm.* Heiddís Smáradóttir, Fiskey Ltd). Previous studies reveal the importance of environmental factors and the physiochemical conditions of the water in which the eggs are subsequently incubated (Brooks *et al.*, 1997; Maeland *et al.*, 2003). The eggs are naturally buoyant at the approproiate salinities and hatch from 72 up to 82 degree days after fertilization, depending on incubation temperatures used (Bergh *et al.*, 2001; Mangor-Jensen *et al.*, 1998). Disinfection of eggs prior to hatching is commonly practised, with glutaric aldehyde recommended for improved hatching yield and larval performance (Olsen *et al.*, 1999b) and up to 60% survival of successfully fertilized eggs commonly obtained with large scale production (*pers. comm.* Heiddís Smáradóttir, Fiskey Ltd).

Hatching is followed by a critical larval yolk sac stage, carried out in specialised incubators designed for the very long incubation period in complete darkness at temperatures between 5 to 9°C for 35-50 days (Bergh *et al.*, 2001; Kjørsvik *et al.*, 2004; Olsen *et al.*, 1999b). Faster development is observed in constant darkness (Howell and Baynes, 2004) and the duration of the yolk sac period depends on temperature as well as the egg size (Falk-Petersen, 2005; Olsen *et al.*, 1999b; Shields, 2001). The larvae are very sensitive to physical and microbial conditions during this stage, and must be handled with care (Falk-Petersen, 2005; Olsen *et al.*, 1999b). Adaptation to lower salinities has been reported for larvae of other flatfish species (Wada *et al.*, 2007). The normal condition of yolk sac larvae is, however, to be neutrally buoyant and Atlantic halibut larvae are relatively sensitive to changes in salinity (Bergh *et al.*, 2001). Approximately 40% survival of the initial stocked population is commonly observed during this stage (*pers. comm*. Heiddís Smáradóttir, Fiskey Ltd.).

Like several other marine fish species, the larvae of Atlantic halibut require live prey animals during the first weeks of exogenous feeding (Næss *et al.*, 1995), with intensive approaches based on enriched nauplii of brine shrimp, *Artemia* spp., commonly practised (Hamre and Harboe, 2008b). Atlantic halibut is characterised by a long period when live feed is offered (Olsen *et al.*, 1999b; Shields, 2001), carried out at approximately 11°C for 60 days (*pers. comm*. Heiddís Smáradóttir, Fiskey Ltd.).

Behavioural responses to light regimes and light conditions are particularly critical for survival and successful larval development during this period (Bergh *et al.*, 2001; Harboe *et al.*, 2009; Le Vay *et al.*, 2007; Olsen *et al.*, 1999b) and the appropriate light conditions and shading effects needed for normal larval behaviour are generally provided by fluor light (300-400 lux) and marine microalgae added to the culture environment in densities ranging between 100-200 M mL^{-1} prior to offering the live prey to larvae in two daily rations (*pers. comm.* Heiddís Smáradóttir, Fiskey Ltd.). Mean survival between 60-70% is commonly obtained during this stage (*pers. comm.* Heiddís Smáradóttir, Fiskey Ltd.).

1.2.2 Live prey quality

Copepods serve as the main pray item for marine fish larvae in their natural environment (Hamre *et al.*, 2005; Koven, 2003; Lee, 2003; Shields *et al.*, 1999a). Due to seasonal variation in abundance and unstable supply, wild-caught copepods represent a limiting factor for stable large scale production, with parasites also representing a potential threat (Guo and Woo, 2009). Commercial production of marine larvae therefore calls for culturing of the live prey commonly practiced at the production sites. Various dietary components have been tested for Atlantic halibut, including extensive or semi-intensive approaches using early stages of marine copepods harvested from the sea, rotifers (*Brachionus* spp.), short-term enriched *Artemia* nauplii and larger *Artemia* juveniles, in addition to various species of marine microalgae added to the culture water (Bergh *et al.*, 2001; Olsen *et al.*, 1999b). The relatively large size of halibut larvae makes *Artemia franciscana* nauplii of suitable size to serve as live prey offered to halibut larvae during the first weeks of exogenous feeding (Conceição *et al.*, 2007; Olsen *et al.*, 1999a). High live feed quality is needed for normal growth, development and survival of halibut larvae (Olsen *et al.*, 1999b) and energy content in addition to the relative quantities and qualities of nutrients are among important characteristics of the live prey (Evjemo *et al.*, 2003; Koven, 2003; Woods, 2003). Prey densities and the viability of the live prey in cold water are furthermore critical for adjustment of the appropriate prey consumption rates (*pers. comm.* Heiddís Smáradóttir, Fiskey Ltd.).

Previous studies report improved growth and survival of larvae offered copepods as compared to rotifers and *Artemia* (Hamre and Harboe, 2008b; Hamre *et al.,* 2002; Næss and Lie, 1998). The lipid content of *Artemia* is typically twice as high as that of marine copepods, but is characterised by low content of non-fatty acid lipids such as wax esters (Evjemo *et al.*, 2003; Olsen *et al.*, 1999b). Halibut larvae have high requirements of *n*-3 highly unsaturated fatty acids (HUFA) such as DHA (docosahexaenoic acid, 22:6 *n*-3), which is the most important individual fatty acid in copepods found in the natural environment of the larvae (Olsen *et al.*, 1999b). The requirements of EPA (eicosapentaenoic acid, 20:5 *n*-3) and ARA (arachidonic acid 20:4 *n*-6) should also be considered for flatfish larvae (Bell *et al.*, 2003; Koven, 2003). Enrichment of the live prey has therefore proved necessary for normal development during production of Atlantic halibut larvae (Bell *et al.*, 2003; Hamre and Harboe, 2008a).

Most types of commercially available *Artemia* spp. may be used for fatty acid enrichment, but high initial EPA in the cysts makes it difficult to achieve the high DHA/EPA ratio needed during enrichment (Bell *et al.*, 2003; Olsen *et al.*, 1999b). Adequate enrichment of *Artemia* can only be obtained with high fat diets that are rich in *n*-3 HUFA, and cysts with a low initial EPA level are therefore preferred for halibut (Olsen *et al.*, 1999b). Variable quality of the *Artemia* from different sources is, however, commonly observed (Lee, 2003; López-Torres and Lizárraga-Partida, 2001) and the fatty acid content of the enriched *Artemia* has been found to be strongly dependent on the enrichment treatment given (Aragão *et al.*, 2004; Olsen *et al.*, 1999b), reflecting the need for adjusted treatment and enrichment methods practised during the culturing (Moraiti-Ioannidou *et al.*, 2007). The high DHA-catabolism in commercially available *Artemia* sources furthermore represent a main problem of using *Artemia* for marine coldwater larvae with high DHA requirements, and more DHA-conservative strains of *Artemia* have therefore been suggested for the production of coldwater species with a long-lasting first feeding stage like halibut (Olsen *et al.*, 1999b). Subsequent problems with metamorphosis of larvae using feeding regimes based only on *Artemia* are commonly observed (Pittman *et al.*, 1998) and insufficient enrichment of the *Artemia* is

still considered one of the main reasons for the high number of deformed juveniles in Atlantic halibut hatcheries in northern Europe (Solbakken *et al.*, 2002).

The marine microalgae commonly used for providing the appropriate shading of the environment may furthermore serve as a feeding source for the live prey as well as larvae, with larval ingestion reported and nutritional benefits suggested (Bergh *et al.*, 2001; Muller-Feuga, 2000; Shields, 2001; van der Meeren *et al.*, 2007). Microalgae stabilize and improve the quality of the rearing water and regulation of the bacterial population, probiotic effects and stimulation of immunity are among other positive functions that have been suggested (Muller-Feuga, 2000). The roles of microalgae in early feeding have, however, not been clearly defined and the nutritional effects are believed to be of minor importance in comparison with the effect on physical parameters, in particular the light regime (Bergh *et al.*, 2001).

1.3 Development and Atlantic halibut from egg through metamorphosis

1.3.1 Eggs and yolk sac larvae

Highly variable success of egg fertilization is observed in batch spawners like the Atlantic halibut, with egg quality and sperm motility depending on the husbandry, nutritional condition and the overall well-being of the parental fish (Babiak *et al.*, 2006; Mazorra *et al.*, 2003; Pavlov *et al.*, 2004; Rurangwa *et al.*, 2004). Temperature and transitions in water temperature furthermore affect spawning rhythm and egg quality (Olsen *et al.*, 1999b). Genetic characteristics are also of importance for offspring viability and larger females commonly provide eggs of higher quality (Ottesen and Babiak, 2007; Ottesen *et al.*, 2009; Pavlov *et al.*, 2004). The females may release up to 16 batches of eggs at 3-4 days intervals, with progressively decreasing egg quality generally produced towards the end of the spawning season (Olsen *et al.*, 1999b; Pavlov *et al.*, 2004). Halibut eggs are very large for their pelagic nature and the larvae hatch at a primitive ontogenetic stage, characterized by a relative large yolk sac and under-devloped organs (Falk-Petersen, 2005; Kjørsvik *et al.*, 2004). A positive relationship has been observed between fertilization success and hatching success, indicative of parental effects on early life history traits (Ottesen and Babiak, 2007). Water quality and

environmental parameters are important during egg incubation and hatching of Atlantic halibut is inhibited by light (Howell and Baynes, 2004). High oxygen concentration may furthermore delay hatching and even cause death of the embryo (Kjørsvik *et al.*, 2004).

The newly hatched yolk sac larvae are transparent and characterized by a simple intestinal tract with mouth and anus closed, no gills, undifferentiated skeleton and incompletely developed eyes, nervous system and sense organs (Bergh *et al.*, 2001; Falk-Petersen, 2005; Kjørsvik *et al.*, 2004). Precursors of vitamin A (carotenoids) are stored in the yolk, with gradual incorporation into the eyes, which are not fully functional until pigmentation is completed approximately 150 degree days after hatching (Falk-Petersen, 2005; Kjørsvik *et al.*, 2004; Rønnestad *et al.*, 1998). Open buccal cavities are observed from 2 days after hatch, connecting the digestive tract with the surrounding seawater, but the mouth does not open until 15-20 days after hatching (Kjørsvik *et al.*, 2004). Increased microvilli density followed by peristaltic movements in the gut is apparent approximately 125 degree days after hatching and halibut larvae seem to be able to degrade and absorb food particles at about 50% yolk sac absorption (Falk-Petersen, 2005). The main organs differentiate and become functional during the yolk sac stage (Falk-Petersen, 2005) and at the time for first feeding, larvae have well-developed heads, brains, eyes and jaws, which are all essential features for survival and further growth (Kjørsvik *et al.*, 2004). Jaw deformation has, however, been identified as a major problem and in halibut larvae jaw deformation is commonly characterized by gaping (Olsen *et al.*, 1999b). This deformation has been associated with abrasion of the head and invasion by microorganisms and resulting in the inability of larvae to feed at the onset of exogenous feeding (Morrison and MacDonald, 1995). Gaping is, however, not considered a problem in large scale halibut farming, with below 10% of larvae suffering this failure (*pers. comm*. Heiddís Smáradóttir, Fiskey Ltd.).

1.3.2 First feeding larvae

After the onset of feeding, timing of developmental events and differentation is controlled by genetic and environmental factors, depending on proper nutrient input and optimal environmental conditions (Falk-Petersen, 2005). Flatfish such as the Atlantic

halibut undergo a dramatic metamorphosis, with a gradual maturation of larval morphology, anatomy and physiology, transforming the pelagic, symmetric larvae into a benthic, cranially asymmetric juvenile (Falk-Petersen, 2005; Kjørsvik *et al.*, 2004; Power *et al.*, 2008). Larval characters disappear and neural change, growth and skeletal change with right sided eye migration, circulatory change and finally establishement of the pigmentation pattern generally represent the sequence of measurable metamorphic events in larvae of the Atlantic halibut, while behavioural changes occur (Falk-Petersen, 2005; Solbakken and Pittman, 2004).

The stomach and the relatively simple intestinal tract characterizing fish in general (Van Loo, 2007) are not fully developed until approximately 700 degree days after the onset of exogenous feeding in halibut larvae (Rønnestad *et al.*, 2007). Still, the capability to degrade and absorb dietary nutrients is sufficient to support fast larval growth, but the physiological constraints of the gut with respect to digestion of cultivated live prey still remain to be elucidated (Rønnestad *et al.*, 2007). This is one of the main reasons why start-feeding has been a bottleneck in the rearing of Atlantic halibut and for problems in developing formulated feeds that are suitable for the larvae (Kjørsvik *et al.*, 2004). In contrast to the high percentage of normally developed larvae fed copepods reared under similar conditions, morphological abnormalities are commonly observed in the production of Atlantic halibut when using *Artemia* (Hamre *et al.*, 2007). These abnormalities include incomplete eye migration and malpigmentation that are considered the two most serious problems in intensive production of Atlantic halibut larvae (Hamre and Harboe, 2008b). Malpigmentation does not impair further growth, but incomplete eye migration often results in loss of the eye due to abrasion against the tank bottom and subsequent infection that may influence normal feeding behaviour and growth in the juvenile and on-growing phase (Pittman *et al.*, 1998; Solbakken *et al.*, 2002). Over 60% of an average population reared according to best practice is in general reported to suffer from this abnormality (Harboe *et al.*, 2009), while incomplete eye migration is observed in only 11% of larvae in a large scale production of Atlantic halibut larvae at Fiskey Ltd. (*pers. comm.* Heiðdís Smáradóttir, Fiskey Ltd.). Incomplete eye migration is therefore considered one of the major

problems in intensive production of juvenile Atlantic halibut, representing a substantial economic loss and a large welfare problem (Harboe *et al.*, 2009).

The complex tissue and organ modifications that accompany flatfish metamorphosis are still relatively poorly characterized and so is the role of hormones and the interplay of abiotic factors in the regulation of the metamorphosis development of larvae (Power *et al.*, 2008). The flatfish metamorphosis is initiated and regulated by the actions of the thyroid hormons, thyroxine (T_4) and triiodothyronine (T_3), with T_3 levels peaking at metamorphic climax during metamorphosis of Atlantic halibut larvae (Galay-Burgos *et al.*, 2008; Koven, 2003). Iodine is an essential part of thyroid hormones (Moren *et al.*, 2006; Power *et al.*, 2008) and aromatic amino acids (phenylalanine and tyrosine) may be specifically required during fish metamorphosis, since they are the precursors of these hormones (Pinto *et al.*, 2009). The iodine concentration in wild zooplankton is many times higher than in *Artemia* (Hamre *et al.*, 2005; Moren *et al.*, 2008; Solbakken *et al.*, 2002) and higher concentrations of T_4, iodine and selenium, and improved eye migration have been observed in Atlantic halibut larvae fed wild zooplankton compared with larvae fed *Artemia* (Solbakken *et al.*, 2002; Sæle *et al.*, 2003). Vitamin A is required for the synthesis of a critical visual pigment and copepods contain higher levels of vitamin A compared to *Artemia* (Koven, 2003). *Artemia* is, however, believed to offer sufficient access to vitamin A precursors to meet the larval requirement and previous studies indicate that *Artemia* may be enriched with iodine up to the levels found in copepods (Hamre *et al.*, 2005; Moren *et al.*, 2006).

Large differences in the concentrations of essential fatty acids and especially DHA, are furthermore found in enriched *Artemia* compared with copepods, and are reflected in the fatty acid composition of the larval body, with improvements in pigmentation of Atlantic halibut larvae achieved by providing the appropriate ratios of DHA/EPA and EPA/ARA in the live prey (Bell *et al.*, 2003; Hamre *et al.*, 2005; Hamre *et al.*, 2002). High levels of *n*-3 HUFA and a low level of ARA in larval diets therefore seem to be necessary for normal pigmentation of Atlantic halibut larvae, whereas energy status and fatty acid composition seem to modulate eye migration (Hamre and Harboe, 2008b). The metamorphosis development and survival of flatfish larvae are also highly affected by environmental salinity levels (Koven, 2003; Wada *et al.*, 2007) and

controlling diurnal light and darkness periods together with a meal-based feeding regime has resulted in the stimulation of eye migration, while continous light regimes may stimulate normal pigmentation and improve survival in production systems for Atlantic halibut (Harboe *et al.*, 2009; Solbakken and Pittman, 2004).

The post-metamorphic phenotype is affected by nutritional, environmental and genetic factors (Lall and Lewis-McCrea, 2007), with many deformities observed during larval and juvenile development of cultured Atlantic halibut (Falk-Petersen, 2005; Lewis *et al.*, 2004; Sæle *et al.*, 2003). Skeletal abnormalities are associated with reduced overall hatchery performance including reduced growth and survival rates and increased susceptibility to stress and diseases (Boglione *et al.*, 2001). Skeletal deformities, however, have not been systematically registered and information on the magnitude and severity of these deformities during intensive production of Atlantic halibut larvae are therefore lacking (Hamre and Harboe, 2008b).

1.3.3 The immune system of larvae

The lymphoid organs in fish include thymus, spleen and head kidney. These organs develop during the yolk-sac stage and are morphologically well developed during the late metamorphic stages in halibut larvae (Patel *et al.*, 2009). The late development of the specific immune system leaves the larvae to rely exclusively on non-specific innate parameters for their protection against environmental bacteria (Lange *et al.*, 2006; Magnadottir, 2006; Vadstein *et al.*, 2004). The innate immune system is therefore believed to be of key importance and stimulation of innate immune parameters is viewed as a promising approach for improved husbandry and health management of Atlantic halibut and other intensively reared fish species. Fish are, however, highly susceptible to environmental parameters and the activity of non-specific immune parameters varies, depending on environmental conditions as well as the nutritional status of the fish and stress conditions (Le Morvan *et al.*, 1998; Robertsen, 1999; Saurabh and Sahoo, 2008).

Innate immune parameters in various forms are found in all multicellular organisms (Zarkadis *et al.*, 2001). The immune system of developing larvae is only partially understood, but the epidermis and mucus surfaces are the first and most

important barriers of fish against their external environment (Kjørsvik *et al.*, 2004). Lysozyme, complement components and antibodies are among molecules found in mucus surfaces and limit the dissemination of infectious agents, whereas nonspecific cytotoxic cells and phagocytes constitute innate cellular immune effectors (Bergsson *et al.*, 2005; Gomez and Balcazar, 2008; Kjørsvik *et al.*, 2004; Magnadottir *et al.*, 2005; Neumann *et al.*, 2001).

Antibodies are generally viewed as a part of the acquired immune response but unspecific natural antibodies have been verified in eggs and larvae of some fish species and are believed to play important roles in innate as well as acquired immune defence (Hayman and Lobb, 1993; Magnadottir, 2006; Olsen and Press, 1997). The ability of natural antibodies to activate the complement system and bind to a variety of different antigens makes them an important part of innate immune protection against pathogens (Chantanachookhin *et al.*, 1991; Lange *et al.*, 2001; Magnadottir *et al.*, 2005). The complement system of teleost fish, like that of higher vertebrates, can be activated through the three complement pathways, all converging to the lytic pathway and leading to opsonization or direct killing of the microorganism (Whyte, 2007). Complement factor C3 is the central component of all three activation pathways and is one of the most abundant serum proteins in addition to being locally synthesized in various tissues of fish (Nakao *et al.*, 2003; Whyte, 2007). Lysozyme (1,4-β-N-acetylmuramidase) specifically hydrolysates the peptidoglycan substructures causing rupture of the cell wall of bacteria and fungi. Lysozyme activity has been validated in fish phagocytes and in the kidney, intestines, spleen, mucuses and sera of various fish species (Saurabh and Sahoo, 2008). Antibacterial properties and the location of the enzyme in areas that are in frequent contact with pathogens, make lysozyme an important factor in protecting fish against bacterial pathogens (Grinde *et al.*, 1988; Saurabh and Sahoo, 2008; Zheng *et al.*, 2007). Lysozyme is furthermore believed to play an important role in nutrient digestion and hence normal larval development (Grinde *et al.*, 1988; Saurabh and Sahoo, 2008).

Maternally transferred proteins with immune functions may play anti-infectious roles in fish larvae (Hanif *et al.*, 2004) and IgM, lysozyme and C3 of parental origin have been detected in larvae of many fish species, as reviewed by Magnadottir (2006)

and Mulero *et al.* (2007). Maternal origin of these components has, however, not been verified for Atlantic halibut larvae.

1.4 Bacteria associated with early production stages

Microbes travel easly between habitats and host in the aquatic environment. The seawater environment host high numbers and a wide variety of bacterial groups, only a few of which have been identified as opportunistic pathogens in fish. Intensive rearing of fish entails high concentrations of nutrients in the form of fish feeds and faeces, that have been suggested to act as bacteria selecting factors supporting the growth of opportunistic pathogens and thereby affecting the establishment of a normal mucosal micorobiota (Hansen and Olafsen, 1999). Hence, organic substrate availability may result in an unpredictable development of the bacterial community that may negatively affect larval growth and survival (Bergh *et al.*, 2001; Eddy and Jones, 2002; Olafsen, 2001; Verner-Jeffreys *et al.*, 2003b). The use of marine microalgae for providing environmental shading has been suggested to positively affect the composition of the intestinal bacterial community of fish larvae as a bacteria selecting factor but may, however, at the same time be carriers of opportunistic pathogens (Liao *et al.*, 2003; Makridis *et al.*, 2006; Olsen *et al.*, 2000).

1.4.1 Normal bacterial community

The outer layers of the fish egg seem highly protective against bacteria (Vadstein *et al.*, 2004), but interactions between bacteria and mucosal surfaces play important roles both at the egg and larval stages of marine fish (Hansen and Olafsen, 1999). Bacterial adhesion and colonization of the egg surface occur within hours after fertilization and the diverse bacterial community which eventually develops on the surfaces of eggs commonly reflects that of the ambient water, but species-specific adhesion at the egg surface may also play a role in development of the egg epimicrobiota (Hansen and Olafsen, 1999).

The microbiota of the gastrointestinal tract is of paramount importance to the health of fish (Olafsen, 2001). During the early developmental stages, the only access from the environment to the larval intestine is through the pseudobranch that are believed to represent the route of entry for the first bacteria that colonise the intestine long before the larvae actually start feeding (Bergh *et al.*, 2001; Olafsen, 2001). The intestinal community of unfed yolk sac larvae is therefore generally believed to reflect that of the ambient water and resemble the egg epimicrobiota (Vadstein *et al.*, 2004), but the yolk sac larvae of halibut have also been suggested to possess a distinct and specific microbiota regardless of geographical origin (Jensen *et al.*, 2004a). The primary intestinal microbiota may persist beyond first feeding (Hansen and Olafsen, 1999), but the microbiota of feeding larvae commonly reflects that of the live prey offered during the first weeks of exogenous feeding (Jensen *et al.*, 2004a; Verner-Jeffreys *et al.*, 2003b).

The natural filtering activity of *Artemia* nauplii will result in large amounts of bacteria in the live prey, with the subsequent risk of contamination with opportunistic and pathogenic groups (Makridis *et al.*, 2000a; Savas *et al.*, 2005). *Vibrio* spp. commonly dominate the bacterial community of *Artemia* spp. (Verner-Jeffreys *et al.*, 2003b) and beneficial effects of members of the *Vibrio* group have been suggested (Austin *et al.*, 1995; Hansen and Olafsen, 1999; Hjelm *et al.*, 2004b; Leyton and Riquelme, 2008; Olafsen, 2001). Many members of the *Vibrio* group have furthermore been recognised as opportunistic pathogens in marine fish species, including the larval stage (Lillehaug *et al.*, 2003; Vadstein *et al.*, 2004). High bacterial numbers are, however, commonly needed for significant mortality caused by *Vibrio spp.* and other opportunistic pathogenic bacteria naturally occurring in the environment (Vadstein *et al.*, 2004).

Early exposure to high bacterial densities may be important for immune tolerance, and thus for the establishment of protective intestinal microbiota (Hansen and Olafsen, 1999). High bacterial densities present in the culture environment, however, represent a major stress and may affect behaviour and overall quality of the poorly developed larvae that are extremely vulnerable against changes in the environment during early developmental stages (Olsen *et al.*, 1999b). Late maturation of the

gastrointestinal tract (Infante and Cahu, 2001) and the specific immune system (Magnadottir *et al.*, 2005) therefore represents significant problems caused by opportunistic and pathogenic bacteria (Gatesoupe *et al.*, 1999; Olafsen, 2001; Vadstein *et al.*, 2004) and the bacterial load and contamination of the production system with opportunistic bacteria has been suggested as one of the main reasons for the overall poor survival of intensively produced marine larvae in general (Olafsen, 2001). The impact of any specific bacterial species or interplay of bacterial groups is, however, poorly defined for marine fish larvae in general and still remains disputable.

The predominant bacterial groups isolated from most fish guts have been aerobes or facultative anaerobes (Bairagi *et al.*, 2002). The anaerobe microbiota of the gastrointestinal tract is believed to play an important function in the digestive capabilities but this part of the gastrointestinal microbiota is poorly characterised in fish (Burr *et al.*, 2005). As pointed out by Bricknell and Dalmo (2005), strategies to control the pathogen load during intensive production of marine fish larvae need to be developed and immuno-prophylactic measures must be addressed further in order to realise the economic potential production of marine fish larvae in general. Successful rearing of early life stages of the Atlantic halibut therefore requires a better understanding of the microbial community associated with various developmental stages.

1.4.2 Analysis of the bacterial community

The microbial diversity of fish intestines has over the past decades primarily been investigated based on culture-based techniques, including isolation of the viable heterotrophic bacteria using nutrient media suited for the isolation of freshwater or marine bacteria (Cahill, 1990; Ringø and Birkbeck, 1999). Various approaches have furthermore been used for group and species identification of the bacterial communities, including serologic characterization, analysis of the chemical composition of the cells and rapid diagnostic tests based on reactions of bacterial enzymes. More recent culture-independent approaches based on homologies and heterogeneities of bacterial 16S ribosomal RNA sequences have revealed many new bacterial communities and species

in the marine environment (DeLong, 2007; DeLong, 2001). Using molecular techniques, microorganisms are grouped according to similarities in their genes, which also reflect their evolutionary relationship (Rudi *et al.*, 2007). Molecular techniques therefore raise the possibility to identify the phylogenetic diversity of microorganisms and their community structure, evolution and taxonomy (Hongoh *et al.*, 2003). Limited information on the different properties of various groups is, however, revealed using molecular techniques and Ranjard *et al.* (2000) suggest that these techniques should be viewed as complementary methods for analysis of complex microbial communities rather than as a substitute for studies of microbial activity and cultivation.

Polymerase Chain Reaction (PCR) amplification using primers directed at universally conserved regions of the bacterial 16S ribosomal RNA (rRNA) gene and subsequent rDNA sequence analysis of the fragments is one of the more sensitive and most widely applied technique for phylogenetic studies of microbial communities in environmental samples (Bernard *et al.*, 2000; Rudi *et al.*, 2007). PCR and subsequent rDNA sequence analysis and fluorescence *in situ* hybridization are among techniques that have been used to characterize the microbial diversity in the intestinal tract of fish during the last decade (Holben *et al.*, 2003; Kim *et al.*, 2007; Namba *et al.*, 2007; Sugita *et al.*, 2005). PCR combined with a denaturing gradient gel electrophoresis (DGGE) has more recently been used for analysis of intestinal communities of fish and often combined with traditional cultivation on selected nutrient media (Hovda *et al.*, 2007; Huber *et al.*, 2004). The PCR products are then fragmented using selected restriction enzymes and labelling used to separate the various length of the fragments in a polyacrylamide gel containing linear gradient of DNA denaturing agent (Muyzer, 1999). The amplified fragments of the 16S rRNA gene are then separated based on the decreased electrophoretic mobility of partially melted double-stranded DNA molecules.

1.5 Manipulating the bacterial community structure

Disease problems and high mortalities during various production stages have led to widespread use of antibiotics in aquaculture through the last few decades (Lillehaug *et al.*, 2003; Miranda and Zemelman, 2002; Sahul Hameed and Balasubramanian, 2000).

Traditional methods to combat infections with antibiotics have been questioned, however (Kesarcodi-Watson *et al.*, 2008; van der Waaij and Nord, 2000), and manipulation of populations or activities of the bacteria colonizing production systems is viewed as providing important therapeutics and improved nutrition and disease prevention in aquaculture (Balcazar *et al.*, 2006; Kesarcodi-Watson *et al.*, 2008; Tinh *et al.*, 2008; Wang *et al.*, 2008). Improved results may be obtained either through direct application of selected bacterial group(s) to the production system using probiotics, or through creating hostile environments for unfavourable groups and/or favouring selected groups using socalled prebiotics (Saulnier *et al.*, 2009), both sharing a unique role in the nutrition of humans (Douglas and Sanders, 2008).

1.5.1 Prebiotics

Selective stimulation of bacterial growth has been achieved through the use of prebiotics, classified as non-digestible food ingredients that beneficially affect the host through selective metabolism in the intestinal tract (Gibson *et al.*, 2004). Selective stimulation of beneficial microbes within the gut microbiota may furthermore directly stimulate immunity, protect against pathogens and facilitate host metabolism and mineral absorption, with prebiotics even exerting their effect beyond the gastrointestinal tract (Saulnier *et al.*, 2009). According to Douglas and Sanders (2008), oligosaccharides and lactulose and possibly several other nondigestible carbohydrates fulfil the criteria of human prebiotics, with functional fibres such as inulin, oligofructose, short-chain fructooligosaccharides and resistant starch exerting beneficial physiological effects in humans. Animals in general do not seem able to hydrolyze beta-glycoside bonds (Van Loo, 2007) and various constituents of yeast, bacteria and plants are among prebiotics favourably affecting various terrestrial species by stimulating growth and activity of a limited number of health-promoting bacteria in the intestine (Burr *et al.*, 2005).

Diet inclusion of β-glucans and alginates may stimulate immune response during early stages of cod and halibut (Skjermo and Bergh, 2004; Skjermo *et al.*, 2006) and immunostimulation, improved disease resistance and health, in addition to enhanced growth performance, has been observed in various fish species as a result of diet

inclusion of inulin and various other oligosaccharides and carbohydrate polymers, as reviewed by Bricknell and Dalmo (2005). Higher diversity of the microbial population of adherent bacteria in the gastrointestinal tract has also been observed following diet inclusion of prebiotics in fish (Bakke-McKellep *et al.*, 2007). Recent studies furthermore indicate increased numbers of selected bacterial groups in the gastrointestinal tract of turbot larvae following diet inclusion of inulin (Mahious *et al.*, 2006), while the prebiotic effect of inulin in seabream has been questioned (Cerezuela *et al.*, 2008). Improved nutrient and energy digestibility of diets following supplementation of prebiotics in diets for fish has furthermore been studied more recently (Burr *et al.*, 2008; Refstie *et al.*, 2006; Xu *et al.*, 2009).

1.5.2 Probiotics

Early definitions describe probiotics as life microbial feed supplements which benefically affect the host by improving its intestinal balance, as reviewed by Verschuere *et al*. (2000). The definitions have been changing with increasing understanding of the mechanisms of action and numerous other and more specific definitions have been proposed (Borchers *et al.*, 2004; Ouwehand *et al.*, 1999). Probiotics are generally described as live microorganism which, through modulating the indigenous intestinal microbiota (Holzapfel and Schillinger, 2002; Rodgers, 2008), confer a health benefit on the host when administered in adequate amounts (Douglas and Sanders, 2008). The modulation can be through maintenance of normal or healthy intestinal microbiota or through competitive exclusion by creating a hostile environment for pathogens by the production of inhibitory compounds (bacteriocins) or by competing for essential nutrients and adhesion sites (Balcazar *et al.*, 2006; Holzapfel and Schillinger, 2002; Ouwehand *et al.*, 1999). Modulation of the intestinal microbiota may furthermore give health effects related to metabolic and nutritional (Ouwehand *et al.*, 1999) as well as immunostimulating effects (Balcazar *et al.*, 2006; Holzapfel and Schillinger, 2002), with an increasing array of other potential modes of action (Ouwehand *et al.*, 1999). The normal microbiota will also influence the innate immune system (Gomez and Balcazar, 2008; Verschuere *et al.*, 2000) and as other constituents of

feeds, ingested bacteria may serve as an exogenous supply of nutrients or essential factors in early life stages (Hansen and Olafsen, 1999).

Improved water quality and larval performance have been achived through the addition of probiotic bacteria to the production systems of marine fish larvae, either to the culture water environment or through bioencapsulation of the live prey animals (Burr *et al.*, 2005; Verschuere *et al.*, 2000; Vine *et al.*, 2006). A successful establishment of a probiotic bacterium(a) within the gut community may prevent the establishment of opportunistic pathogens entering through the gastrointestinal tract of fish, as reviewed by Tinh *et al*. (2008) and Verschuere *et al*. (2000). Repeated treatments may, however, be needed, whereas beneficial effects have been observed without successful colonization of the probiont in the gastrointestinal lumen (Planas *et al.*, 2006). Stimulation of selected immune parameters as a result of probiotic treatment has furthermore been demonstrated in various fish species, as reviewed by Gatesoupe (2008). Studies on the use of probiotics during early production stages of Atlantic halibut larvae are limited, however, and the most recent studies found were published by Makridis *et al.* (2001) and Ottesen and Olafsen (2000), as reviewed by Vine *et al*. (2006). Two doses of probiotic-bioencapuslated *Artemia* were insufficient to influence predictably the intestinal species composition of halibut larvae over a 10-day period (Makridis *et al.*, 2001), while *Vibrio salmonicida* increased halibut larval survival by 14.6% (Ottesen and Olafsen, 2000).

The importance of the origin of the selected probiotics with respect to survival and competitive attachment to intestinal mucus has been stressed (Gatesoupe, 1999; Vine *et al.*, 2006) and whereas yeasts are commonly isolated from the gastrointestinal tract of fish (Sreedevi and Rosamma, 2008), the benefits of yeasts during early feeding of fish larvae have been suggested (Gatesoupe, 2007). The selection of probiotic bacteria requires various *in vitro* screening experiments, which assay for the production of antagonist compounds, the growth in and attachment to the intestinal mucus and the production of beneficial compounds such as vitamins, fatty acids and digestive enzymes, as reviewed by Vine *et al*. (2006). Tinh *et al*. (2008), however, point out that effects observed *in vitro* will not necessarily be reflected *in vivo* and probiotics for use in aquaculture are generally selected based on their ability to produce beneficial and/or

antimicrobial metabolites, with a generally poor characterization of the gastrointestinal microbiota of the fish (Burr *et al.*, 2005). Hence, the impact of any specific bacterial species or interplay of bacterial groups during various developmental stages of fish is in general poorly defined (Burr *et al.*, 2005) and the *in vivo* mechanisms of action of probiotics in aquaculture largely remain to be unravelled (Tinh *et al.*, 2008). More detailed studies and a general understanding of the microbial community associated with rearing of marine larvae are therefore needed to potentially enhance the effectiveness of probiotic supplementation.

1.6 Immunostimulation

Early definitions describe immunostimulants as chemical compounds that activate the immune system of animals and render them more resistant to infections by viruses, bacteria, fungi and parasites, as reviewed by Verschuere *et al*. (2000). A definition more recently proposed describes immunostimulants as naturally occurring compounds that modulate the immune system by increasing the host's resistance against diseases (Bricknell and Dalmo, 2005). Modulation of the immune response may furthermore be obtained through diet inclusion of probiotics and prebiotics (Douglas and Sanders, 2008), where surface molecules have been implicated as key factors involved in immunomodulation (Saulnier *et al.*, 2009).

1.6.1 The mechanism of immunostimulation

Evidence suggests that the non-specific defence of vertebrates has evolved towards recognition of structurally conserved microbial polymers like fungal cell wall β-glucans, bacterial lipopolysaccharides and peptidoglycan, bacterial DNA and viral double-stranded RNA (Robertsen, 1999). Stimulation commonly occurs through repeated structure sequences leading to recognition through Toll-like receptors (TLRs) that are pattern recognition receptors conserved from insects to mammals and that play important roles in host immune defence (Connor *et al.*, 2009; Dalmo and Bogwald, 2008; Jault *et al.*, 2004; Jiang *et al.*, 2007). Immunostimulants may directly initiate

activation of the innate defence mechanisms that result in production of anti-microbial molecules, such as viral double-stranded RNA that has been shown to enhance the resistance of fish against infections with viral pathogens, and fungal β-glucans and peptidoglycan polymers that have been shown to enhance the resistance of fish against infections, as reviewed by Robertsen (1999) and Dalmo and Bogwald (2008). These immunostimulants are often obtained from bacterial sources and more recently also from brown or red algae and terrestrial fungi, with their effects depending on the type of receptors carried by the stimulated cells (Bricknell and Dalmo, 2005).

1.6.2 Immunostimulation in fish

Immunostimulation is viewed as a promising method for improved production performances during intensive production of marine larvae, and a variety of different immunostimulants have been studied in fish and shrimp aquaculture, as reviewed by Bricknell and Dalmo (2005) and Sakai (1999). β-glucans, high-mannuronic acid (high-M) alginate, nucleotides, nisin and *Photobacterium damsela* bactrim have been tested as immunostimulants in turbot, while reports of immunostimulation in Atlantic halibut are lacking, as reviewed by Bricknell and Dalmo (2005).

Several studies suggest that dietary inclusion of hydrolysed protein may compensate for any lack in the larval capacity for processing dietary intact proteins and increased absorption rates of peptides have been observed in halibut larvae with increasing degree of hydrolysis (Rønnestad *et al.*, 2007). Furthermore, diet inclusion of protein hydrolysates, like other constitutents of fish feeds, will affect the intestinal microbiota (Savas *et al.*, 2005). Protein hydrolysates derived from fish muscle have also been reported to enhance a non-specific immune response in fish (Liang *et al.*, 2006). Besides serving as easily digested nutrition for larvae, fish protein hydrolysates may therefore represent an important measure for improved production performances during intensive production of marine fish larvae through modification of the intestinal microbiota and/or through stimulation of non-specific immune components. Diet inclusion of hydrolysed fish proteins has therefore received increased attention during the early production stages of marine fish species. The effects on diet utilization, growth

and survival have been studied in Atlantic halibut larvae (Kvåle *et al.*, 2009; Nordgreen *et al.*, 2009; Tonheim *et al.*, 2005), while studies involving stimulation of non-specific immune parameters and resistance to infection are lacking, as pointed out by Kotzamanis *et al.* (2007) and Liang *et al*. (2006).

Despite reports of successful stimulation of innate immune parameters of marine larvae, the effects of immunostimulation on the developing immune system of larvae are not fully documented (Bricknell and Dalmo, 2005). The timing of treatment is critical and it is generally accepted that the feeding of immunostimulants to immunologically mature fish is beneficial, while early treatment may affect normal development of the stimulated cells. Overstimulation has also been reported and immune tolerance has been experienced as a result of long-term stimulation in fish, as reviewed by Bricknell and Dalmo (2005).

2 AIMS OF THE STUDY

The heavy mortalities commonly experienced during early production stages of marine larvae are believed to be partly due to an unfavourable environment that encourages the growth of bacteria and unpredictable development of the bacterial community. Identification of structural developments of the bacterial community related to larval success will improve our knowledge and help develop preventive measures for improved production performance. The aims of this study were to describe the development of bacterial numbers and the bacterial community structure during the early production stages of halibut larvae and to study bacterial structural development in relation to various treatments. Additionally, the aims included studying the effects of the various treatments on the bacterial community and the overall success of halibut eggs and larvae.

The specific aims were:

1) To analyse and describe the "normal" bacterial community of halibut larvae at a commercial hatchery site.
2) To compare cultivation and molecular methods for analysis of the bacterial community.
3) To study the relationship between the bacterial community structure and the overall quality and growth performance of first feeding halibut larvae.
4) To study the effects of reduced organic load in the culture environment on bacterial growth within the production system.
5) To investigate the effects of microbial manipulation of eggs and larvae on the bacterial community structure, using bacterial isolates dominating the cultivable community of overall successful first feeding larvae.
6) To study the effects of bioencapsulation of the live prey using a fish protein hydrolysate on the bacterial community structure and selected parameters of the unspecific immune system of larvae.

3 MATERIALS AND METHODS

Experiments performed *in vivo* were carried out in commercial-size production units at Fiskey Ltd., producing 35-55% of the annual global production of halibut juveniles (Hjalteyri, IS-601 Akureyri, Iceland). A continuous production with manipulation of the photoperiod results in three distinct groups spawning at advanced and delayed periods compared to the normal group. The extended yolk sac and first feeding incubation periods result in an ongoing production more or less the whole year around, with the normal spawning group supplying the largest part of the annual production of larvae. Highly variable survival rates and overall success are commonly observed for larvae of different parental origin. Treatments were therefore carried out in production units containing first feeding larvae of a common silo origin (sibling tank units).

3.1 Production methods, survival and quality of halibut eggs and larvae

Seawater used for the production was pumped from 70 m depth in the fjord, approximately 100 m from the shore. The seawater was then run through a water treatment system, including protein skimming, UV disinfection, ozone control and biological filtration (International Aqua-Tech Ltd., United Kingdom). Egg fertilization was carried out at 5°C using minimum light (red light). Eggs (~1 L) and milt (10-20 mL of a mixture from two males) were added to separate holders containing ~1/2 L of seawater and gently mixed before mixing together, followed by incubation in the dark at 5°C for 10 min. Up to 6 L of fertilized eggs were then transferred to 0.25 m^3 tanks followed by incubation in the dark at 5.0–5.3°C. Also, ~5 mL aliquots (≥ 40 eggs) were transferred to flasks containing ~150 mL of 5°C seawater and incubated for 24 h at 5°C for calculating the ratio of successfully fertilized eggs. This was done by arranging the eggs on a transparent plastic plate and confirming cell division under light in a stereoscope. The diameter of ten eggs from each flask was furthermore measured for determination of the average egg size (2.8–3.2 mm). The water flow was adjusted to 1 L min-1 during the first 24 h when aeration was added and the water flow adjusted according to the number of litres of eggs transferred to each incubator (3-5 L min-1). Following incubation for 14 days, the eggs were collected and transferred to seawater

containing 400 ppm glutaraldehyde for surface disinfection at 5.0-5.2°C for 7 min prior to transferring to 10 m^3 silos where the eggs hatched. Egg survival was calculated based on litres of eggs transferred to each incubator, with each litre estimated to hold 40,000 eggs. The yolk sac larvae were held at 5.0–5.3 °C in the dark for ~50 days prior to transferring to first feeding tanks (3.5 or 7.0 m^3) where enriched *Artemia franciscana* nauplii were offered to larvae as described in ***Paper I***. The survival and quality of unfed yolk sac larvae were estimated at transfer to first feeding tanks and larval success through first feeding was calculated at the onset of weaning unto formulated feed at ~60 days post onset of first feeding (dpff), as described in ***Paper I***. Larval growth was monitored as described in ***Paper I***. The last samples for evaluation of larval growth in individual incubators were collected between 40-65 dpff. The growth curves were therefore extrapolated to 55 and 65 dpff when comparing the final weight of larvae from various incubators during the individual periods studied.

The *Artemia* nauplii (Instar III stage) were obtained from decapsulated *Artemia* cysts that were hatched during a 24 h incubation followed by 24 h enrichment as described in detail in ***Paper III***. The necessary environmental shading during the first weeks of offering exogenous feed to larvae was provided by marine microalgae until 2001, when studies on the use of inorganic clay were initiated, as described in ***Paper II***. The microalgae were cultured semi-continuously and added to the tank water environment prior to offering enriched *Artemia* to larvae in two daily feedings. The concentration of inorganic clay was visually adjusted in order to achieve similar shading effects throughout the first feeding period as described in ***Paper II***. Since 2003 inorganic clay has been used for environmental shading during the whole period of production, including the periods presented in ***Papers I***, ***III*** and ***IV***.

3.2 Sample collection and preparation

Samples were collected and prepared as described in details in ***Papers I*** and ***II***. Briefly, tank water was collected in sterile glass bottles that were opened below the water surface. Eggs and larvae were collected using a mesh net and then transferred to sterile jars filled with water from the respective tank. The eggs were then collected by gently pouring

through a sieve, with homogenization in a tenfold dilution of peptone-seawater. The larvae were killed with an overdose of Hypnodil added to the jars (51µg mL^{-1}) and then collected by gently pouring through a sieve followed by surface sterilization in 0.1% benzalkonium chloride solution to remove surface bacteria (Grisez et al., 1997). Following rinsing and enumeration, the larvae were homogenized in a tenfold dilution of peptone-seawater in order to free bacteria from the gastrointestinal tract, as described in detail in ***Paper I***. The live prey was harvested into a mesh net and homogenized following thorough rinsing under running tap water for 2 min, as described in ***Paper I***.

3.3 Sample analysis

Analysis of samples included enumeration of colony forming units (CFU) on selected nutrient media as described in detail in ***Paper I***. Briefly, serial tenfold dilutions of the homogenates and culture water samples were prepared in peptone-seawater within 4 h post collection. Samples (100 µl) were then surface plated in duplicates-triplicates for enumeration of total hetertrophic aerobes on marine agar (MA; Difco) and presumptive *Vibrio* bacteria on thiosulphate citrate bile salt sucrose agar (TCBS; Difco) following incubation for 5-7 days at 15°C. Results are expressed as the mean numbers of CFU mL^{-1} and $larvae^{-1}$ and in each g wet weight of halibut eggs and the live prey. Ten-twelve colonies were then randomly picked from MA plates of selected samples and sub-cultured to ensure purity of the isolates followed by presumptive identification through morphological and biochemical characterization as described in ***Paper I***.

Analysis of the bacterial community also included assessment of different phylotypes through DGGE analysis of PCR amplified fragments of the 16S rRNA gene. Following sample preparation, 1 mL aliquots of sample homogenates were preserved in an ultralow freezer at -80°C for later analysis using DGGE (***Papers I***, ***III*** and ***IV***) and measuring the IgM concentration in larvae (***Paper IV***). Five larvae from each sample were furthermore embedded in a plastic tube as described in ***Paper IV***, covered in a TissueTek medium and the blocks then frozen in liquid nitrogen prior to storing at -80 °C for later analysis using immunohistochemical methods.

3.3.1 Extraction of bacterial DNA from samples

DNA was extracted from thawed homogenates of samples using the PureGene® Extraction Kit (Gentra D-70KA, USA) as described in detail in ***Paper I***, with modifications made throughout the period presented in ***Paper III***. DNA was also extracted from CFU growth from selected samples, collected from MA plates containing 200-300 CFU plate^{-1}. The growth was dissolved in ~1mL of dH_2O, vortexed and then left at room temperature for 5-10 min prior to centrifugation and adding the cell lysis solution. The reaction solutions were warmed at room temperature and then thoroughly mixed for dissolving precipitations if developed during storage. Various measures were carried out in order to increase the recovery of bacterial DNA in the samples, including additional treatment of samples to ensure satisfactory homogenization and breakdown of larval tissue. The phenol-chloroform extraction method described by (Bonaiti *et al.*, 2006) was compared with the PureGene Extraction Kit (Gentra) for removing proteins from the nucleic acid in difficult samples such as samples of the live prey. Incubation with Proteinase K (Gentra, USA) was furthermore tested, without obtaining improved results and extraction for 5 min at 80°C without Proteinase K was selected for all studies. Incubation with RNase A solution was varied between 15 and 55 min, with 15 min at 37°C selected for all studies. For inadequate protein precipitation, the supernatant was taken from just above the pellet and the tube placed in -20°C for another minute prior to repeating the protein precipitation step. The length of the drying time for the precipitated DNA was varied between 5 and 60 min, with 20 min selected for all studies. Higher volumes of hydration solution were added to large pellets to further dilute the DNA solution. Following incubation the samples were left overnight at room temperature with gentle shaking prior to amplification of the DNA or storing at -80°C until further analysis. The concentration of DNA in samples was routinely measured using Quant-it dsDNA BR assay in QUBIT (Invitrogen).

3.3.2 The PCR amplification and DGGE analysis

The PCR amplification was first carried out with the universal eubacterial primers 341F-GC and 534R (TAG Copenhagen) in a "touchdown" PCR, with initial annealing

temperature set at 65°C and a 1°C decrease for each circle to 55°C and a total of 40 cycles. In order to avoid the observed co-amplification of 18S rDNA from larvae and their live prey, a new set of primers was selected in order to specifically amplify the 16S rDNA gene. PCR annealing temperatures of 65°C and 55°C were furthermore tested instead of the "touchdown" amplification. The PCR products were routinely examined on 2% agarose gels, visualised by staining with 1.0 mg mL^{-1} ethidium bromide. The DGGE analysis was performed as described in detail in ***Paper I***, with further adjustments of methods carried out throughout the period in order to increase the differentiation of bands resolving in the gel when analysing various types of samples. An overview of the PCR and DGGE methods used in the studies is given in Table 1. A relative mobility standard was run in every gel, consisting of PCR products from laboratory subcultures and type strains with various GC content and hence resolving in different regions of the gel (Table 2).

Table 1. An overview of primers and DGGE methods used in the studies.

	Paper I	**Papers III and IV**[§]	**Paper I**[§]
Universal eubacterial primers (TAG Copenhagen)	533F-GC and 787R	533F-GC and 787R	341F-GC and 534R
Annealing temperature	55°C	55°C	touchdown (65-55°C)
Denaturing gradient (urea-formamide)	40-70%	30-60%	30-60%
Acrylamide-bis concentration	6%	8%	10%
Electrophoresis (60V, 20 mA at 60°C)	14 h	14 h	10 h

[§] Including additional results not presented in the attached papers

Table 2. Bacterial isolates included in the relative mobility standard run in every gel.

Product labelling	**Product identification**	**GeneBank accession number and similarity**	**Presented**
A	*Pseudoalteromonas elyakovii*	AB000389 (99%)	Paper I, III, IV [§]
B[2]	*Vibrio splendidus*	AJ874364 (100%)	Paper I, III [§]
B[1]	*Vibrio* spp. V798	DQ146994 (100%)	Paper III, IV [§]
C	*Marinovum algicola*	DSM 10251	Paper I, III, IV [§]
D	*Shewanella baltica*	CP000891 (99%)	Paper I, III, IV [§]
E	*Streptomyces* spp.	EU 257269 (99%)	Paper I[§]
F	*Staphylococcus aureus*	DSM 20231	Paper I[§]

[§] Including additional results not presented in the attached papers

Subsequent staining of the gels with SYBR Gold (Invitrogen) nucleic acid staining was followed by imaging under UV light as described in detail in ***Paper I***. A small core was excised from bands of interest for another PCR reaction as described in detail in ***Paper I***. The 16S rDNA fragment were then identified by sequence analysis (Matis-Prokaria ohf.), alignment with the BLAST programme to closest matches in GeneBank and related databases, and further phylogenetic identification using the Ribosomal Database Project II programme (Wang et al., 2007). The quality of the re-amplified bands was routinely checked by DGGE and the profiles were highly reproducible. The 254 bp products excised from the gels, however, generally proved too short for a decisive species identification using 16S rDNA sequencing. Hence, the products were identified to the family or genus level and only occasionally to the species level. Furthermore, surface sterilization of larvae will not simultaneously exclude bacterial DNA existing on the surface of larvae and the PCR-DGGE pattern is therefore referred to as the bacterial community associated with surface sterilized larvae.

Isolates dominating the cultivable bacterial community of selected samples were furthermore identified by partial 16S rDNA sequencing as described in detail in ***Paper I***.

3.4 Selection of bacteria for microbial manipulation

A total of 540 bacterial isolates were collected for selection of isolates for bacterial treatment of halibut eggs, and larvae through bioencapsulation of the live prey during the studies presented in ***Paper I***. The isolates were randomly selected from the dominating cultivable bacterial community of unfed yolk sac larvae from 9 incubators (24 isolates) and first feeding larvae from 4 incubators resulting in overall poor larval success (132 isolates) and 9 incubators resulting in overall high larval success (384 isolates), as described in detail in ***Paper III***. The 384 isolates that dominated the intestinal community of overall successful larvae were screened for their *in vitro* inhibitory properties against two fish pathogens (*Vibrio anguillarum* and *Aeromonas salmonicida* subspecies *salmonicida*) and the 132 isolates that dominated the intestinal community of larvae from incubators resulting in an overall poor success, as described in ***Paper III***. Thirteen

isolates were then selected, based on growth inhibiting activity against the test isolates as well as identification only in overall successful larvae and not in larvae from any of the incubators resulting in overall poor larval success. Partial 16S rDNA sequencing of the thirteen isolates revealed six groups and representative isolates from three of the groups were then selected, as described in ***Paper III***. The three isolates were represented by *Vibrio* spp. V798 (GeneBank accession number DQ146994), *Pseudoalteromonas elyakovii* (GeneBank accession number AB000389) and *Vibrio splendidus* (GeneBank accession number AJ874364).

3.5 Treatment of halibut eggs and larvae

Freeze-dried preparations containing equal CFU numbers of the three selected isolates were used for repeated treatment of halibut eggs through adding the bacterial mixture to the culture water of eggs at 0, 6 and 13 days post fertilization (10^7 cfu L^{-1}) and bathing the eggs in the bacterial mixture (10^9 cfu L^{-1}) for 5 min immediately prior to transfer to yolk sac incubators, as described in detail in ***Paper III***. Treatment of larvae was carried out through bioencapsulation of the live prey by immersion in the bacterial mixture for 30 min (10^9 cfu L^{-1}) prior to offering to larvae in the first of two daily feedings at 0, 1, 17 and 18 dpff (24 h *Artemia*), as described in ***Paper III***. On the selected treatment days, the remainders of the bioencapsulated live prey cultures were incubated for additional 8 h (32 h *Artemia*) and then bathed in the bacterial mixture for 30 min (10^7 cfu L^{-1}) prior to offering to larvae in the second daily feeding, as described in ***Paper III***.

First feeding larvae were furthermore treated using a fish protein hydrolysate, as described in detail in ***Paper IV***. The peptides were manufactured through hydrolysis of fillets from Pollock (*Pollachius pollachius*) in a chilled environment using the Hultin-Process method (Kristinsson and Rasco, 2000). Treatment of larvae was carried out through bioencapsulation of the live prey where the hydrolysate was added to the fatty acid enrichment medium of the *Artemia* (0.02 g L^{-1}) followed by enrichment of the live prey cultures for 24–32 h prior to offering to larvae in both daily feedings (24 h and 32 h *Artemia*). Larvae were offered the bioencapsulated live prey from the onset of and through the first ~60 days of exogenous feeding, as described in ***Paper IV***.

In both experiments described in ***Paper III*** and ***Paper IV***, the same enrichment media, though without the addition of bacteria or fish protein hydrolysate, were used for enrichment of the life feed offered to the control group of larvae in sibling tank units.

3.6 Immunological analysis

The concentration of IgM in larval extracts was measured using an enzyme linked immunosorbent assay (ELISA) as described in ***Paper IV***. Briefly, thawed homogenates were further homogenized followed by centrifugation and collection of the aqueous phase. Flat-bottom microtiter plates were coated with rabbit anti-halibut IgM antibodies, unbound sites saturated and dilutions of larval extracts then incubated on the plate in quadruples, with phosphate buffered saline (PBS) containing 0.2% w/v bovine serum albumin and 0.05% v/v Tween® 20 (PBSA-Tween) used as a negative control and purified halibut IgM as a positive control on each plate. Incubation with the capture antibody (mouse anti-halibut IgM) and the secondary antibody (alkaline phosphatase conjugated rabbit anti-mouse IgG) was followed by incubation with the substrate solution and optical density then measured at 405 nm. The value of negative controls was subtracted from the reading and the final values calculated as the mean of four repeats for each sample.

Five larvae were furthermore collected from each treatment group on individual sampling dates, for immunohistological analysis of IgM, C3 and lysozyme as described in detail in ***Paper IV***. Briefly, larvae were cryosectioned and the sections then fixed in acetone before blocking of endogenous peroxidase activity and active sites for protein binding. Incubation with the primary antibodies (rabbit anti-halibut IgM, mouse anti-halibut C3 or rabbit anti-cod lysozyme) was followed by washing and incubation with horseradish perioxidase (HRP) or fluorescent labelled secondary antibodies. Incubation with HRP-labelled secondary antibodies was followed by incubation with a substrate solution prior to counterstaining with hematoxylin. The sections were then rinsed prior to mounting and microscopic analysis.

3.7 Selection of samples for analysis

Samples of yolk sac and first feeding larvae were collected from all production units of the normal spawning group of 2006 during the studies presented in ***Paper I***, with the first eggs brought in during May and the last larvae transferred to weaning in October. During the studies presented in ***Paper II***, samples of first feeding larvae and their culture water were collected from a large number of incubators during the production of three spawning groups with environmental shading provided by marine microalgae (1999-2001) and three spawning groups with environmental shading provided by inorganic clay (2001-2002). Samples from individual incubators, including sibling tank units, were collected at weekly intervals throughout first feeding and a minimum of seven production units were used for calculating the difference between the two groups at individual sampling dates, as described in ***Paper II***. In the studies presented in ***Paper III***, a mixture of the three selected bacterial isolates was used for treatment of halibut eggs and the live prey of larvae from the normal spawning group of 2007 and the treatments repeated in the normal spawning group of 2008. In ***Paper IV***, treatments through bioencapsulation of the live prey using a Pollock protein hydrolysate were carried out on first feeding larvae from the normal spawning group of 2007.

Samples of eggs were collected at weekly intervals throughout the two week incubation period (***Paper III***) and samples of unfed yolk sac larvae were collected immediately before the onset of exogenous feeding (***Papers I***, ***III*** & ***IV***). First feeding larvae were collected from individual incubators after 1 and 5 weeks of feeding (***Paper I***), or at weekly intervals throughout the first feeding period (***Papers II***, ***III*** & ***IV***). Samples of enriched *Artemia* were collected at approximately weekly intervals throughout the individual periods studied. Samples were furthermore collected from the culture water of eggs (***Paper III***) and larvae (***Paper II***). An overview of the samples analysed during the studies presented in ***Papers I-IV*** is given in Table 3.

Table 3. An overview of samples analysed during the studies presented in Papers I-IV. The number of incubators included in the individual studies is indicated by "n".

	Paper I	Paper II	Paper III	Paper IV
Halibut eggs †	*n.d.*	*n.d.*	n=15	*n.d.*
culture water †	*n.d.*	*n.d.*	n=15	*n.d.*
Yolk sac larvae §	n=9 (0 dpff)	*n.d.*	n=5	n=1
culture water §	*n.d.*	*n.d.*	*n.d.*	*n.d.*
First feeding larvae	n=14 (7 and 36 dpff)	n=25 (weekly for ~8 weeks)	n=8	n=2
culture water	*n.d.*	n=25 (weekly for ~8 weeks)	*n.d.*	*n.d.*
Live prey (24 h *Artemia*)	n=14	n=48	n=25	n=14

n.d. not detected
dpff, days post onset of first feeding
† samples collected 0, 7 and 14 days post fertilization
§ samples collected immediately prior to the onset of exogenous feeding (0 dpff)

Additional samples not listed in the attached papers were as follows. Seawater for the production was collected at approximately weekly intervals during the studies presented in ***Paper II*** (2001-2003). The bacterial community of first feeding larvae was analysed after two weeks of feeding during the studies presented in ***Paper I*** (2007). Additional results not presented in ***Paper II*** included a more recent study of the bacterial community of larvae and their culture water after one and two weeks of feeding in addition to samples of the live prey collected through the period (2009). The study was carried out in sibling tank units containing larvae of a common silo origin, with environmental shading provided by marine microalgae or inorganic clay. Furthermore, the bacterial community structure of single newly hatched *Artemia* cysts and during various developmental stages of the *Artemia* were studied (2008).

3.8 Statistical analysis

Data were analysed using SigmaStat® release 3.5 (Systat Software Inc. CA 94804-2028 USA). The normality of the data distribution was analysed using the Kolmogorov-Smirnov test. The numbers of CFU are expressed as mean ± S.D. in a minimum of two samples, with each sample analysed in duplicate-triplicate. The numbers of CFU in larvae from individual production units were compared using a *t*-test or one-way

ANOVA, with Kruskal-Wallis one way analysis of variance and all pairwise multiple comparison procedures (Dunn's Method) used when normality failed. In ***Paper II***, a *t*-test was used to analyse the difference in CFU numbers in larvae or their culture water from a minimum of seven production units from each group at the various sampling dates, using the Mann-Whitney rank sum test when normality failed.

Survival of first feeding larvae in individual incubators was compared using the χ^2 test (***Papers I***, ***III*** and ***IV***). Growth of first feeding larvae was extrapolated to 55 or 65 dpff and a *t*-test then used to compare the weight and success of larvae in individual production units, using the mean values and the standard error of the mean for all production units during each period studied. A *t*-test was also used to compare the survival of successfully fertilized eggs in the two groups studied in ***Paper III***.

Regression analysis was used to analyse the relationship between parameters found to be linearly related. Pearson's correlation was used to analyse the relationship between bacterial numbers at various sampling points and larval growth, survival and metamorphosis characteristics used for evaluation of larval success. In ***Paper IV***, the concentration of IgM in larvae from the two groups was expressed as the mean ± S.D. and a *t*-test was used to compare the two groups at individual sample points.

Differences were considered statistically significant when $p<0.05$.

4 RESULTS

This section summarizes the main results from analysis of the bacterial community during intensive production of halibut larvae and its relation to survival, growth and quality of first feeding larvae described in ***Paper I*** (2006-2007). Isolates dominating the cultivable bacterial community in the gastrointestinal tract of overall successful larvae were then selected for manipulation of the bacterial community of eggs and larvae described in ***Paper III*** (2007-2008). ***Paper II*** describes the effects on the cultivable bacterial community and larval survival when using inorganic clay (2001-2002) instead of marine microalgae (1999-2000) for providing the environmental shading necessary during the first weeks in feeding. ***Paper IV*** describes the effects on the bacterial community and selected parameters of the unspecific immune system of larvae when offering peptide-enriched live prey to larvae during the first weeks of exogenous feeding (2007-2008).

The general focus is on the bacterial community of larvae during a period of elevated larval mortalities commonly observed during the first two weeks in feeding, with bacteriological analysis of the culture water included in ***Paper II*** and halibut eggs included in ***Paper III***. An analysis of bacterial numbers in seawater used for the production and the bacterial community structure during various developmental stages of the live prey is furthermore presented, followed by a detailed analysis of larval growth and mortalities in selected incubators during the periods studied in ***Papers I*** and ***III***.

4.1 Analysis of the bacterial community

Analysis of the cultivable bacterial community of larvae and their live feed revealed approximately one log-unit lower numbers of CFU on TCBS compared with MA. Lower and more variable numbers of CFU were observed on TCBS when analysing the bacterial community in the culture water of larvae (***Paper II***, Figures 1 and 3). A variable percentage of the cultivated bacterial isolates did not grow upon sub-culturing or responded poorly in the morpho-physiological tests used. This was more commonly observed for isolates dominating the cultivable community of yolk sac larvae (8 - 100% of isolates in individual samples) as compared to first feeding larvae (8 - 34% of isolates

in individual samples) (***Paper I***).

The PCR-DGGE method was adjusted throughout the studies in order to specifically amplify the 16S rRNA gene and for improving the differentiation of products when analysing various types of samples. The PCR reaction was first carried out using the 341F-GC and 534R eubacterial universal primers, resulting in the overall dominance of products identified as eukaryotic 18S rDNA (results not shown). Selection of a new primer set, 533F-GC and 787R (Griffiths *et al.*, 2001), and the appropriate denaturing gradient and acryamide-bis concentration for the gels, resulted in specific amplification of bacterial 16S and high diversity of products when analysing samples of larvae and their live prey. Varying the denaturing gradient furthermore gave different separations of products dominating the community structure in the various types of samples represented by halibut eggs, larvae, their culture water and their live prey. Staining of DGGE gels was crucial because of the variable sensitivity of the staining methods commonly used. Staining with SYBR Gold nucleic acid stain resolved a clear view of bacterial isolates present in lower relative quantities that were not visible by staining with ethidium bromide (results not shown). Also, sequencing of the ~250 bp products excised from the DGGE gels commonly proved to be too short for a decisive identification of bacterial species and more than one group was occasionally identified in products excised from the gels. The products were therefore generally identified to the family or genus level and only occasionally to the species level. This was reflected, for example, by the DGGE profiles of the two *Vibrio* isolates, *Vibrio* spp. V 798 and *Vibrio splendidus* that were selected for microbial manipulation of halibut eggs and larvae during the studies presented in ***Paper III***. When using the denaturing concentration and acrylamide gradient selected during the studies presented in ***Paper III***, the two *Vibrio* isolates were not separated within the gels (product "B" in the relative mobility standard run in every gel), while *P. elyakovii* (product "A" in the relative mobility standard run in every gel) was clearly distinguished from the *Vibrio* product.

The cultivable bacterial community of first feeding larvae was dominated by various products identified as *Vibrio* spp., *Shewanella* spp. and *Photobacterium* spp. and in unfed yolk sac larvae by *Pseudoalteromonas* spp., *Marinomonas* spp. and *Shewanella* spp. (***Paper I***).

4.2 Halibut eggs and larvae

4.2.1 Halibut eggs

An analysis of the bacterial community of halibut eggs is presented in ***Paper III***. Briefly, the CFU numbers in samples from eggs increased by 2 to 3 log-units during the first week following fertilization (***Paper III***, Table 3). A PCR-DGGE analysis revealed different and less diverse profiles in eggs as compared to the product pattern observed in larvae (***Paper III***, Figures 1 and 2). *Tenacibaculum ovolyticum* was identified as a part of the dominating bacterial community and *Pseudoalteromonas*/*Lacinutrix* and *Marinomonas* spp. co-dominated the bacterial community of eggs from most incubators. Significantly increased numbers of CFU on TCBS were observed in eggs that were treated with a mixture of three selected bacterial isolates as compared to untreated eggs (***Paper III***, Table 3). Products identical to the isolates used for bacterial treatment were detected only in samples of treated eggs and in no samples collected from the control incubators (***Paper III***, Figure 1). The results therefore indicated a successful establishment of the bacterial isolates during the egg stage, however, without affecting egg survival (***Paper III***, Table 3). Surface disinfection of eggs prior to hatching resulted in a significant reduction in the numbers of CFU only in eggs from the control group (***Paper III***, Table 3). The *Vibrio* isolates used for bacterial treatment may furthermore have been removed by the surface disinfection (***Paper III***, Figure 1).

4.2.2 Yolk sac larvae

A positive relationship was observed between the CFU numbers in the gastrointestinal tract of unfed yolk sac and jaw deformation of larvae during the studies presented in ***Paper II*** (Figure 1).

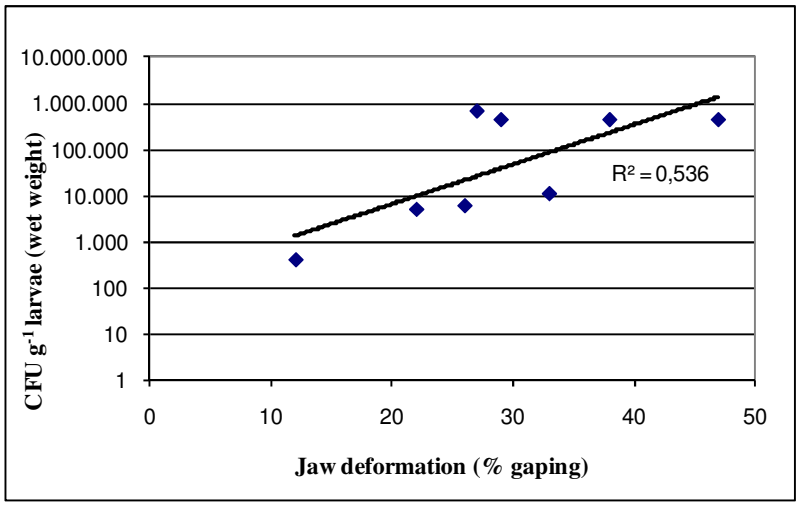

Figure 1. Bacterial numbers and jaw deformation amongst unfed yolk sac larvae. Shown is the relationship between colony forming units (CFU) observed on marine agar and the incidence of jaw deformation (%) amongst yolk sac larvae estimated immediately prior to the onset of exogenous feeding.

The DGGE profiles of yolk sac larvae included in the experiments presented in ***Papers III*** and ***IV*** are shown in Figure 2, with variable profiles observed in larvae from different incubators. *Pseudoalteromonas* spp. and *Vibrio* spp. (GeneBank accession number AM941184) dominated the bacterial community of larvae from all incubators, with a product identified as *Stenotrophomonas* spp. observed in most larval samples (Figure 2 and Table 4). A distinct product identified as *Stenotrophomonas* spp. was, however, observed only in larvae originating from eggs that were bathed in the bacterial mixture immediately prior to hatching (Figure 2, product # 12). A product identified as *Shewanella* spp. was furthermore observed only in larvae originating from eggs that were bathed repeatedly in the bacterial mixture prior to hatching (Figure 2, product # 5).

The bacterial community of unfed yolk sac larvae was studied in detail and the results presented in ***Paper I*** (***Paper I***, Figure 4 and Tables 1 and 2). Results from the analysis of the bacterial community of yolk sac larvae during the studies presented in ***Papers I***, ***III*** and ***IV*** are summarized in Table 5.

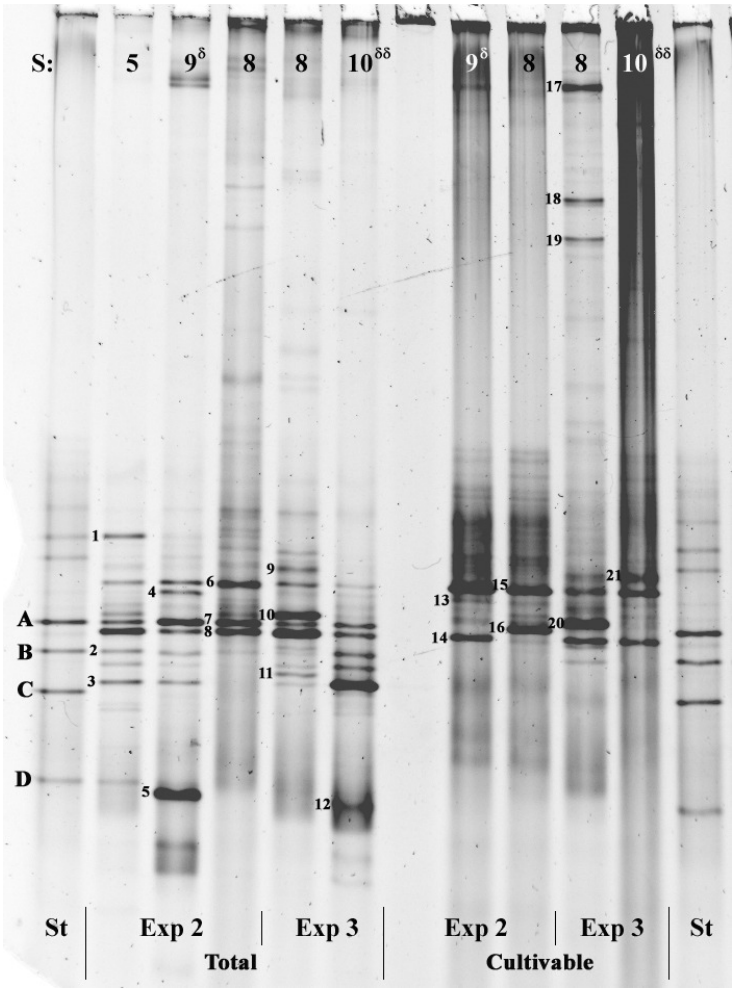

Figure 2. DGGE profiles of unfed yolk sac larvae.
Shown are the profiles in a pool of ~150 yolk sac larvae collected from individual incubators immediately prior to the onset of exogenous feeding. Incubator numbers (S) indicate the silo-origin of larvae, with "δ" indicating larvae originating from eggs that were bathed repeatedly in the bacterial mixture during incubation, or immediately prior to hatching only (δ δ). Also shown are the profiles of the cultivable bacterial community from the same samples and relative mobility standards (St), represented by *Pseudoalteromonas elyakovii* (A), *Vibrio splendidus* and *Vibrio* spp. (B), *Marinovum algicola* (C) and *Shewanella baltica* (D). Labelling indicates bands that were excised from the gel and identified by sequence analysis (Table 4). The gel contains 30-60% urea-formamide denaturing gradient and 8% acrylamide-bis. The bacteria are represented by 16S rDNA sequences covering the variable region 4 of the gene (bp 533-787).

Table 4. Identification of products excised from the gel shown in Figure 2.
Groups identified as a part of the bacterial community of surface sterilised yolk sac larvae immediately prior to the onset of exogenous feeding. The products identified are represented by 16S rDNA sequences covering the variable region 4 of the gene (bp 533-787).

Product	BLAST identification	Division (% similarity)	GeneBank link
1	Firmicutes bacterium clone	Firmicutes (98%)	EF188711
2	*Pseudoalteromonas* spp.	γ-Proteobacteria (100%)	EI935099
3	*Stenotrophomonas* spp.	γ-Proteobacteria (100%)	EU054384
4	*Pseudoalteromonas* spp.	γ-Proteobacteria (100%)	EU935099
5	*Shewanella* spp.	γ-Proteobacteria (99%)	EU931529
6	Uncultured *Vibrio* spp.	γ-Proteobacteria (99%)	AM941184
7	*Pseudoalteromonas* spp.	γ-Proteobacteria (100%)	EU935099
8	*Pseudoalteromonas* spp.	γ-Proteobacteria (100%)	EU935099
9	*Oceanospirillaceae* bacterium	γ-Proteobacteria (95%)	FM162973
10	*Pseudomonas* spp.	γ-Proteobacteria (99%)	EU770268
11	Bacilli bacterium clone	Firmicutes (99%)	EF703477
12	*Stenotrophomonas* spp.	γ-Proteobacteria (100%)	EU054384
13	*Marinomonas* spp.	γ-Proteobacteria (98%)	FJ196055
14	*Pseudoalteromonas* spp.	γ-Proteobacteria (100%)	FJ169999
15	*Marinomonas* spp.	γ-Proteobacteria (99%)	FJ196055
16	*Pseudoalteromonas* spp.	γ-Proteobacteria (98%)	FJ200650
17	*Pseudoalteromonas* spp.	γ-Proteobacteria (96%)	FJ169999
18	*Pseudoalteromonas* spp.	γ-Proteobacteria (97%)	FJ169992
19	*Shewanella* spp.	γ-Proteobacteria (98%)	DQ011269
20	*Shewanella* spp.	γ-Proteobacteria (100%)	DQ011269
21	Proteobacterium symbiont of *Osedax* spp.	Proteobacteria (99%)	DQ911542
	Uncultured Antarctic bacterium clone	99%	DQ906761
	Oceanospirillaceae bacterium	98%	AJ717296

Table 5. Bacterial community of unfed yolk sac larvae.
Analysis of the bacterial community of surface sterilized yolk sac larvae collected immediately prior to the onset of first feeding. Also shown are larval survival (%) and the ratio of larvae with jaw deformation (%). Incubator labelling indicates the silo origin of larvae.

Incubator #	Survival (%)	Jaw deformation (%)	CFU larvae^{-1} (MA)§	CFU larvae^{-1} (TCBS)§	Products in DGGE (#)	Dominating groups identified by 16S rDNA sequences covering the variable region 4 of the gene (bp 533-787) †, ††
Paper I (untreated larvae)						
11	37	24	$1.5*10^4 \pm 10^2$	< 3	≤ 5	*Vibrio*††; *Pseudoalteromonas* †† *Aeromonas*/*Citrobacter*††
12	41	11	$2.1*10^3 \pm 10^2$	< 3	≤ 5	*Pseudoalteromonas* ††; *Aeromonas*/*Citrobacter*†† *Ralstonia*††; *Pelomonas aquatica*/*Shewanella*††
1	33	29	$1.1*10^3 \pm 10^2$	< 3	≤ 5	*Pseudoalteromonas* ††; *Aeromonas*/*Citrobacter*†† *Marinomonas*††; *Pelomonas aquatica*/*Shewanella*†
2	77	18	$3.9*10^5 \pm 10^4$	$1.8*10^3 \pm 10^2$	5-10	*Pseudoalteromonas* ††; *Aeromonas*/*Citrobacter*† β-Proteobacterium/*Citrobacter*† ; *Marinomonas*† *Pelomonas aquatica*/*Shewanella*††
3	27	20	$2.1*10^3 \pm 10^2$	< 3	5-10	*Pseudoalteromonas* ††; *Aeromonas*/*Citrobacter*†† *Ralstonia*† ; *Marinomonas*††; *Marinobacter*†† *Pelomonas aquatica*/*Shewanella*††
4	27	22	$3.0*10^1 \pm 0$	< 3	10-15	*Vibrio*††; *Pseudoalteromonas* ††; *Marinomonas*†† *Aeromonas*/*Citrobacter*†† ; *Ralstonia*†† *Marinobacter*†† ; *Pelomonas aquatica*/*Shewanella*††
5	43	40	$1.8*10^4 \pm 10^3$	$1.2*10^3 \pm 10^2$	5-10	*Marinomonas*††; *Aeromonas*/*Citrobacter*†† *Marinobacter*†; *Pelomonas aquatica*/*Shewanella*††
6	69	25	$6.1*10^1 \pm 10^1$	< 3	10-15	*Vibrio*††; *Pseudoalteromonas* ††; *Marinomonas*†† *Aeromonas*/ *Citrobacter*†† ; *Ralstonia*†† *Pelomonas aquatica*/*Shewanella*†† β-Proteobacterium/*Citrobacter*††
Paper III (Experiment 2), Paper IV						
5	44.1	14	$6.3*10^3 \pm 10^3$	< 1	5-10	*Vibrio* spp.†† ; Firmicutes bacterium clone† *Pseudomonas* spp.†† ; *Pseudoalteromonas* spp. † *Pseudoalteromonas* spp.†† ; *Stenotrophomonas* spp. †
6	40.3	30	$5.3*10^3 \pm 10^3$	$4.5*10^2 \pm 10^1$	< 5	*n.d.*

Table 5. ***Continued***

8	13.8	4	$1.0*10^{6} \pm 10^{4}$	< 1	< 5	*Vibrio* spp.[†] ; *Pseudoalteromonas* spp. [†]
9[δ]	33.7	9	$1.1*10^{6} \pm 10^{5}$	$1.1*10^{1} \pm 10^{0}$	5-10	*Vibrio* spp.[††]; *Shewanella* spp. [†]; *Pseudoalteromonas* spp. *Pseudoalteromonas* spp.[††] ; *Stenotrophomonas* spp.[††]
Paper III (Experiment 3)						
8	52	15	$1.6*10^{2} \pm 10^{1}$	$1.7*10^{1} \pm 10^{0}$	5-10	*Vibrio* spp. [††]; *Pseudoalteromonas* spp.[††] *Pseudomonas* spp [†]; *Stenotrophomonas* spp.[††] Bacilli bacterium clone[†]; *Pseudoalteromonas* spp. [††] *Oceanospirillaceae* bacterium[†]
10[δδ]	31	2.5	$1.4*10^{2} \pm 10^{0}$	< 1	5-10	*Vibrio* spp.[††]; *Stenotrophomonas* spp. [†] *Stenotrophomonas* spp.[††] ; *Pseudoalteromonas* spp.[††] *Pseudoalteromonas* spp.[††]

n.d., not detected ;

§ *colony forming units, mean values ±S.D. in duplicate-triplicate samples on marine agar (MA) and thiosulphate citrate bile salt sucrose agar (TCBS)*

[†] *Products excised from the gel and identified by 16S rDNA sequences covering the variable region 4 of the gene (bp 533-787)*

[††]*Presumptively identical bands excised from parallel run samples and identified by 16S rDNA sequences covering the variable region 4 of the gene (bp 533-787)*

[δ] *Larvae originating from eggs that were bathed repeatedly in the bacterial mixture prior to hatching*

[δδ] *Larvae originating from eggs that were bathed in the bacterial mixture immediately prior to hatching*

A considerable variation in CFU numbers (up to 4 log units) was observed in unfed yolk sac larvae from various incubators, within individual periods as well as when comparing the different periods studied (Table 5). The numbers of CFU on MA were 10^1–10^5 CFU larvae^{-1} and <3–10^3 CFU larvae^{-1} were observed on TCBS during the studies presented in ***Paper I*** (2006-2007). The highest numbers of CFU were found in larvae originating from incubators resulting in the highest survival and significantly higher than in larvae from other production units studied during this period (Table 5 and ***Paper I***, Figure 1). Low numbers of CFU were, however, observed in larvae from a production unit resulting in the second highest larval survival observed during the period, indicative of a lack of relationship between CFU numbers and the success of unfed yolk sac larvae. Excluding the most successful production unit of the period, however, revealed a positive correlation between the incidence of jaw deformation and the CFU numbers observed in larvae (***Paper I***).

High numbers of CFU were observed in larvae originating from untreated eggs as well as eggs that were bathed repeatedly in the bacterial mixture prior to hatching during the studies presented in ***Paper III*** (2007-2008) (***Paper III***, Table 5, Experiment 2). A single bathing of eggs in the bacterial mixture immediately prior to hatching resulted in low numbers of CFU observed on MA and similar to the numbers observed in untreated larvae (Table 5, Experiment 3). Hence, the overall results do not indicate increased numbers of cultivable bacteria in yolk sac larvae as a result of bacterial treatment during the egg stage. No relationship was observed between the numbers of CFU in larvae and the survival or success of unfed yolk sac larvae during this period.

The DGGE profiles indicate a highly variable bacterial community structure of unfed yolk sac larvae originating from different incubators (Table 5). A product identified as a β-Proteobacterium/*Citrobacter* was observed only in larvae from two incubators during the studies presented in ***Paper I*** and both resulted in high survival rates (Table 5, Silos 2 and 6). A comparison of the DGGE profiles of larvae from incubators resulting in similar survival but containing the highest and lowest ratio of jaw deformation observed during the period revealed the identification of *Pseudoalteromonas* spp. and *Ralstonia* spp. as a part of the bacterial community of larvae with a low ratio of gaping, and *Marinomonas* spp. and *Marinobacter* spp. in

larvae with a high ratio of gaping (Table 5, Silos 12 and 5). A product identified as *Shewanella* spp. was furthermore observed only in larvae originating from bacteria-treated eggs during the studies presented in ***Paper III***. A product identified as *Shewanella* spp. was, however, identified in unfed yolk sac larvae originating from untreated eggs during the studies presented in ***Paper I*** (***Paper I***, Figure 4 and Table 2). Products identified as *Stenotropomonas* spp. were furthermore identified in unfed yolk sac larvae from most incubators during the studies presented in ***Papers III*** and ***IV*** but in none of the samples analysed during the studies presented in ***Paper I*** (Table 5). Also, various products identified as *Vibrio* spp. may be identified in larvae from only some of the incubators included in the studies presented in ***Paper I***, but in contrast in larvae from all incubators included in the studies presented in ***Papers III*** and ***IV*** (Table 5).

Overall the results indicate variable bacterial communities of larvae during the different periods studied, without any relationship observed between larval survival and the DGGE product diversity.

4.2.3 First feeding larvae

Highly variable numbers of CFU were observed in larvae from individual incubators at various sampling dates, with higher numbers generally observed with environmental shading provided by marine algae as compared to inorganic clay during the studies presented in ***Paper II*** (***Paper II***, Table 1). Lower CFU numbers were observed in larvae already after the first day in feeding with environmental shading provided by inorganic clay as compared to marine microalgae (***Paper II***, Figure 2 A and B). The highest numbers of CFU on MA were reached towards the end of the first feeding period with the addition of marine microalgae, but already after two weeks in feeding with the addition of inorganic clay (***Paper II***, Figure 2 A). Larval survival was neither affected by the method selected for providing environmental shading, nor was it correlated with bacterial numbers observed in larvae after two weeks in feeding (***Paper II***, Table 1). A positive relationship was, however, observed between larval survival and the numbers of CFU on TCBS observed in larvae after two weeks in feeding with environmental shading provided by inorganic clay (R^2=0.629).

The studies presented in ***Paper II*** were carried out during 1999-2002. An analysis of the bacterial community of larvae was studied more closely during 2009, in sibling tank units containing larvae of a common silo origin and with the necessary environmental shading provided by marine microalgae or inorganic clay (Table 6). Relatively lower numbers of CFU were observed in first feeding larvae studied during 2009 compared to the study presented in ***Paper II*** (Table 6). In both studies, lower numbers of CFU were observed in larvae with environmental shading provided by inorganic clay as compared to marine microalgae (Table 6 and ***Paper I***, Table 1). In the study presented in ***Paper II,*** only the cultivable bacterial community was studied, but a DGGE analysis was also carried out during the study performed in 2009. The DGGE profiles revealed a somewhat different pattern in larvae with environmental shading provided by marine microalgae as compared to inorganic clay, both in larvae collected after one and two weeks in feeding (Figure 3 and Table 7). Variable DGGE profiles were furthermore observed in samples of the live prey collected through the period (Figure 3). Various products identified as *Vibrio* spp. dominated the bacterial community of larvae from both groups. A product identified as *Pseudoalteromonas* spp. co-dominated the bacterial community, with *Marinomonas* spp. observed only after two weeks in feeding. A distinct product identified as *Marinomonas* spp. in addition to a *Shewanella* spp. were observed only in larvae from tanks with environmental shading provided by marine microalgae.

Table 6. Bacterial numbers in first feeding larvae.
Numbers of colony forming units (CFU) on MA and TCBS after 7 and 14 days in feeding (dpff), with environmental shading provided by inorganic clay or marine microalgae. CFU numbers during the studies presented in Paper II show mean values ±S.D. in duplicate samples collected from ≥7 incubators from each group, while the CFU numbers during the studies carried out in 2009 show mean values ±S.D. in duplicate samples collected from sibling tank units containing larvae of a common silo origin.

	Marine microalgae				**Inorganic clay**			
	7 dpff		**14 dpff**		**7 dpff**		**14 dpff**	
	CFU (MA) [§]	**CFU (TCBS)**	**CFU (MA)** [§]	**CFU (TCBS)** [§]	**CFU (MA)** [§]	**CFU (TCBS)**	**CFU (MA)** [§]	**CFU (TCBS)**
Paper II	$1.0*10^5 \pm 10^5$	$1.4*10^4 \pm 10^4$	$7.2*10^4 \pm 10^4$	$1.5*10^4 \pm 10^4$	$2.2*10^5 \pm 10^5$	$1.1*10^4 \pm 10^4$	$9.4*10^4 \pm 10^4$	$2.6*10^3 \pm 10^3$
2009	$3.0*10^4 \pm 10^3$	$1.3*10^2 \pm 10^2$	$2.7*10^4 \pm 10^3$	$8.5*10^3 \pm 10^3$	$4.1*10^3 \pm 10^2$	$0.8*10^1 \pm 10^0$	$2.3*10^4 \pm 10^2$	$0.8*10^3 \pm 10^2$

§ Colony forming units (CFU), mean values ±S.D. on marine agar (MA) and thiosulphate citrate bile salt sucrose agar (TCBS)

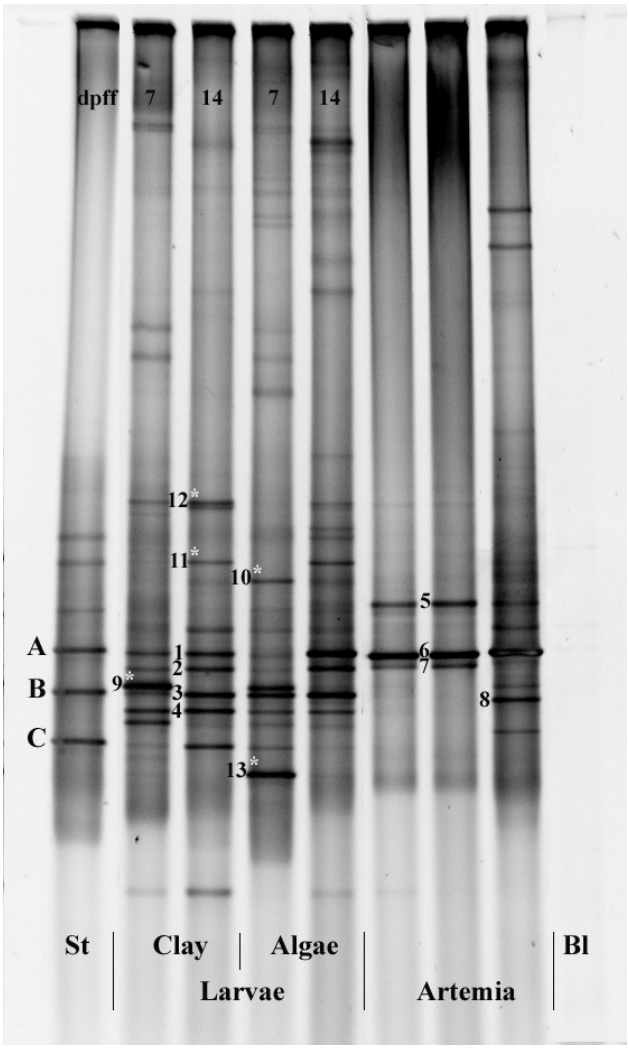

Figure 3. DGGE profiles of first feeding larvae.
Shown are the profiles in a pool of 75-150 surface sterilized larvae collected 7 and 14 days post onset of first feeding (dpff), with environmental shading provided by inorganic clay (Clay) or marine microalgae (Algae). Also shown are samples of the live prey (*Artemia*) collected through the period. Included in the gel is a negative control (Bl) and relative mobility standards (St.), represented by *Pseudoalteromonas elyakovii* (A), *Vibrio splendidus* and *Vibrio* spp. (B) and *Marinovum algicola* (C). Labelled products indicate bands that were excised from the gel and identified by sequence analysis (Table 7). Products labelled with an asterisk (*) indicate presumptively identical bands excised from parallel run samples identified by sequence analysis. The gel contains 30-60% urea-formamide denaturing gradient and 8% acrylamide-bis. The bacteria are represented by 16S rDNA sequences covering the variable region 4 of the gene (bp 533-787).

Table 7. Identification of products excised from the gel shown in Figure 3.
Groups identified as a part of the bacterial community of halibut larvae 7 and 14 days post onset of first feeding. Products labelled with an asterisk (*) were identified from bands excised from parallel run samples. The products identified are represented by 16S rDNA sequences covering the variable region 4 of the gene (bp 533-787).

Product	BLAST identification	Division (% similarity)	GeneBank link
1	*Pseudoalteromonas* spp.	γ-Proteobacteria (98%)	FJ404756
2	*Vibrio* spp.	γ-Proteobacteria (96%)	AM989925
	Vibrio vulnificus	γ-Proteobacteria (96%)	EF546308
3	*Vibrio* spp.	γ-Proteobacteria (100%)	FJ596492
	Vibrio splendidus	γ-Proteobacteria (100%)	FM954973
4	Uncultured bacterium clone	(100%)	FJ456712
	Vibrio spp.	γ-Proteobacteria (100%)	AM913931
5	*Vibrio* spp.	γ-Proteobacteria (99%)	FJ99031
	Vibrio harveyi	γ-Proteobacteria (99%)	FJ605242
6	*Vibrio harveyi*	γ-Proteobacteria (99%)	FJ605242
	Vibrio spp.	γ-Proteobacteria (99%)	FJ799031
7	*Vibrio rotiferianus*	γ-Proteobacteria (99%)	FM204863
	Photobacterium phosphoreum	γ-Proteobacteria (99%)	EU281142
	Marine bacterium	(99%)	EU268276
	Vibrio parahaemolyticus	γ-Proteobacteria (99%)	FJ594056
8	*Alteromonas* spp.	γ-Proteobacteria (95%)	DQ530517
9*	*Alteromonas* spp.	γ-Proteobacteria (96%)	EU600749
	Glaciecola spp.	γ-Proteobacteria (96%)	EU268077
10*	*Marinomonas* spp.	γ-Proteobacteria (94%)	FJ457290
	Oceanospirillales bacterium	γ-Proteobacteria (94%)	DQ810540
11*	Uncultured marine bacterium	(95%)	EU338370
	Marinomonas spp.	γ-Proteobacteria (95%)	FJ457290
	Oceanospirillales bacterium	γ-Proteobacteria (95%)	DQ810540
12*	*Psychrobacter* spp.	γ-Proteobacteria (100%)	FJ039851
13*	*Shewanella* spp.	γ-Proteobacteria (97%)	FJ626842

Analysis of the bacterial community of larvae after one and five weeks in feeding is presented in ***Paper I***. Briefly, the bacterial community was dominated by various products identified as *Vibrio* spp. and *Pseudomonas* spp. after one week in feeding and by *Vibrio* spp., *Pseudoaltermonas* spp./*Blastomonas* spp., *Shewanella* spp. and *Acidovorax* spp. after five weeks in feeding, without any relationship observed between larval success and the numbers of CFU or the DGGE profiles of larvae. Highly variable

DGGE profiles were also observed in larvae from different incubators after two weeks in feeding (Figure 4, Table 8), without any relationship observed between the bacterial community, and the overall success of larvae at the end of the first feeding period (***Paper I***, Figure 1).

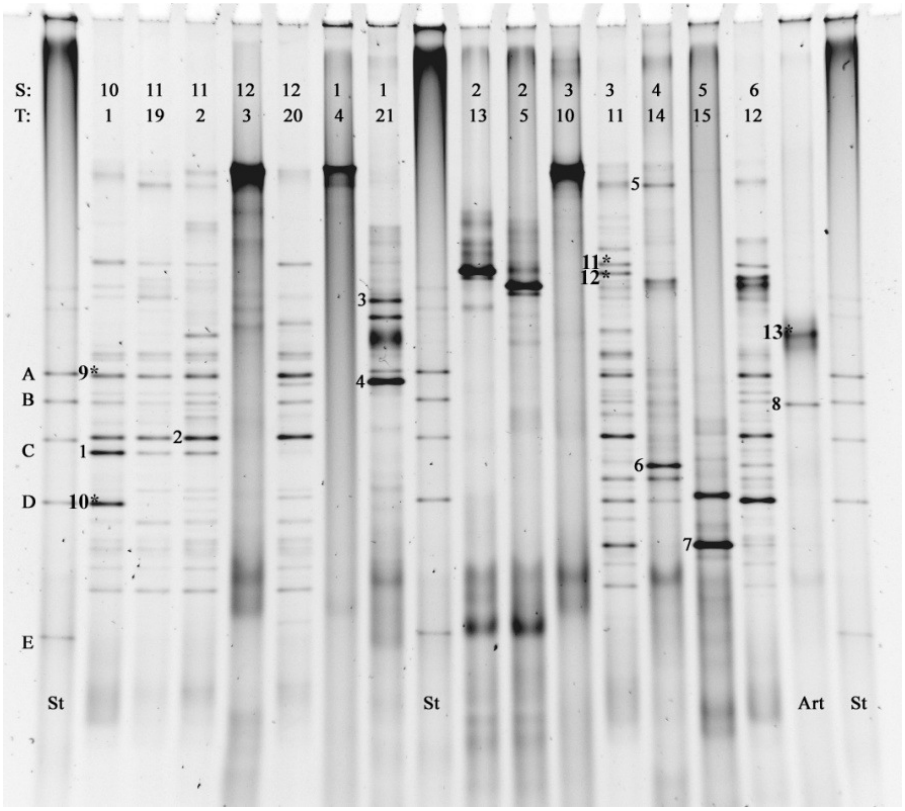

Figure 4. DGGE profiles of first feeding larvae during the studies presented in Paper I. Shown are the profiles in a pool of ~75 surface sterilized larvae collected from individual incubators after two weeks of feeding. Incubator numbering indicates the silo-tank (S-T) origin of larvae. Also shown is the profile in a sample of the live prey (Art) and relative mobility standards (St), represented by *Pseudoalteromonas elyakovii* (A), *Vibrio splendidus* and *Vibrio* spp. (B), *Marinovum algicola* (C), *Shewanella baltica* (D) and *Streptomyces* spp. (E). Labelling indicates bands that were excised from the gel and identified by sequence analysis (Table 8). Labelling denoted with an asterisk (*) indicates presumptively identical bands excised from parallel run samples identified by sequence analysis. The gel contains 40-70% urea-formamide denaturing gradient and 6% acrylamide-bis.The bacteria are represented by 16S rDNA sequences covering the variable region 4 of the gene (bp 533-787).

Table 8. Identification of products excised from the gel shown in Figure 4.
Groups identified as a part of the bacterial community of larvae after two weeks in feeding. Labelling denoted with an asterisk (*) indicates presumptively identical bands excised from parallel run samples identified by sequence analysis. The products identified are represented by 16S rDNA sequences covering the variable region 4 of the gene (bp 533-787).

Product	BLAST identification	Division (% similarity)	GeneBank link
1	*Stenotrophomonas* spp.	γ-Proteobacteria (100%)	GQ184202
	Pseudomonas spp.	γ-Proteobacteria (100%)	FJ605176
2	*Acidovorax* spp.	β-Proteobacteria (100%)	FJ605421
3	Uncultured *Bacilli* bacterium	(100%)	EF700048
4	*Enterococcus* spp.	γ-Proteobacteria (100%)	FJ513935
	Tenacibaculum spp.	Flavobacteria (98%)	AB274770
5	*Flexibacter aurantiacus*	Flavobacteria (98%)	AB078044
	Cytophaga spp.	Sphingobacteria (98%)	EF492014
6	*Arhodomonas* spp.	γ-Proteobacteria (99%)	AJ315984
	Saccharospirillum impatiens	γ-Proteobacteria (99%)	AJ315983
7	*Raoultella terrigena*	γ-Proteobacteria (100%)	GQ169108
	Citrobacter spp.	γ-Proteobacteria (100%)	GQ205112
	Enterobacter spp.	γ-Proteobacteria (100%)	FJ357823
8	*Vibrio* spp.	γ-Proteobacteria (99%)	EU177084
9*	*Blastomonas* spp.	α-Proteobacteria (99%)	AB242676
10*	*Shewanella* spp.	γ-Proteobacteria (100%)	EU075118
11*	*Psychrobacter* spp.	γ-Proteobacteria (100%)	EU836736
12*	*Acinetobacter* spp.	γ-Proteobacteria (99%)	EU848481
13*	*Marinomonas* spp.	γ-Proteobacteria (99%)	EU052766

The effects of bacterial treatment of halibut eggs and offering bacteria-treated live prey to larvae are presented in ***Paper III***. Briefly, bathing halibut eggs in the bacterial mixture (10^7-10^9 CFU L^{-1}) revealed a successful establishment of the isolates on eggs, however, without affecting egg survival. Products identical to the isolates used for bacterial treatment were observed in treated eggs only and not in eggs from any of the control incubators included in the study (***Paper III***, Figure 1). Products identical to the isolates used for bacterial treatment were furthermore identified in treated as well as untreated first feeding larvae and their live prey (***Paper III***, Figures 2 and 3). Offering bacteria-treated *Artemia* to larvae, however, resulted in significantly improved larval survival in the two experiments that were carried out (***Paper III***, Table 4). Grazing of the

Artemia in a mixture of the bacterial isolates did not result in increased numbers of CFU and similar DGGE profiles were observed for treated and untreated *Artemia* (***Paper III***, Figure 2). A product identified as *Pseudoalteromonas* spp. was, however, identified in samples of treated *Artemia* only and products identified as *Corynebacterium* spp. and *Moraxella* spp. only in samples of untreated *Artemia* (***Paper III***, Figure 2 and Table 5).

Repeated addition of a fish peptide hydrolysate or high numbers of bacteria to the culture water of larvae resulted in an accumulation of organic materials inside the incubator's walls and a subsequent increase in the numbers of CFU was observed in the culture water (results not shown). Bioencapsulation through grazing of the live prey in seawater containing the peptide hydrolysate or a mixture of the bacterial isolates was therefore selected for further treatment of larvae as described in ***Papers III*** and ***IV***. An analysis of the bacterial community of larvae is summarized in Table 9, with focus on two weeks post onset of first feeding, when elevated larval mortalities are commonly observed.

Table 9. Bacterial community of first feeding larvae.
Analysis of the bacterial community of surface sterilized larvae after ~two weeks in feeding. Also shown are larval survival (%) and success of metamorphosis (%), estimated at transfer of larvae to weaning six weeks later. Incubator labelling indicates the silo-tank origin of larvae.

Incubator #	Survival (%)	Incomplete eye migration (%)	Incomplete pigmentation (%)	CFU larvae^{-1} (MA)§	CFU larvae^{-1} (TCBS)§	Products in DGGE (#)	Dominating groups identified by 16S rDNA sequences covering the variable region 4 of the gene (bp 533-787) †, ††
Paper I							
10-1	83	10	12	$8.1*10^4 \pm 10^3$	$5.0*10^3 \pm 10^3$	15-20	*Vibrio* spp. ; *Acidovorax* spp. ; *Shewanella* spp. Uncultured *Bacilli* ; *Blastomonas* spp. *Psychrobacter* spp. *Stenotrophomonas* spp./*Pseudomonas* spp.
11-2	64	14	7	$1.1*10^6 \pm 10^4$	$8.7*10^4 \pm 10^3$	15-20	*Vibrio* spp. ; *Blastomonas* spp. ; *Psychrobacter* spp. *Marinomonas* spp. ; *Acidovorax* spp.† *Flexibacter*/*Cytohpaga* spp. *Stenotrophomonas* spp./*Pseudomonas* spp.
11-19	94	2	13	$1.2*10^6 \pm 10^4$	$2.9*10^4 \pm 10^3$	15-20	*Vibrio* spp. ; *Acidovorax* spp. ; *Blastomonas* spp. *Psychrobacter* spp. ; *Flexibacter*/*Cytohpaga* spp. *Stenotrophomonas* spp./*Pseudomonas* spp.
12-3	59	3	12	$7.4*10^5 \pm 10^4$	$1.3*10^5 \pm 10^3$	< 5	*n.d.*
12-20	71	0	7	$2.7*10^6 \pm 10^4$	$1.4*10^5 \pm 10^3$	15-20	*Blastomonas* spp. ; *Acidovorax* spp.; *Vibrio* spp. *Psychrobacter* spp. *Enterococcus* spp./*Tenacibaculum* spp.
1-4	59	0	15	$1.4*10^5 \pm 10^3$	$1.1*10^4 \pm 10^2$	< 5	*n.d.*
1-21	62	3	15	$1.4*10^5 \pm 10^3$	$6.8*10^3 \pm 10^3$	5-10	*Marinomonas* spp. ; Uncultured *Bacilli* † *Enterococcus* spp./*Tenacibaculum* spp.†
2-5	60	6	4	$1.1*10^5 \pm 10^3$	$1.4*10^3 \pm 10^1$	< 5	*Psychrobacter* spp.
2-13	48	26	7	$0.8*10^5 \pm 10^3$	$2.2*10^3 \pm 10^2$	5-10	*Psychrobacter* spp.
3-10	70	0	15	$6.9*10^5 \pm 10^4$	$6.7*10^5 \pm 10^5$	< 5	*n.d.*
3-11	57	2	11	$1.4*10^6 \pm 10^4$	$3.2*10^4 \pm 10^3$	15-20	*Vibrio* spp. ; *Acidovorax* spp. ; *Psychrobacter* spp. *Shewanella* spp. ; *Blastomonas* spp. *Acinetobacter* spp. ; *Flexibacter*/*Cytohpaga* spp. *Raoultella* spp./*Citrobacter* spp/*Enterobacter* spp.

Table 9. ***Continued***

4-14	86	6	9	$3.0*10^5 \pm 10^4$	*n.d.*	5-10	*Flexibacter*/*Cytohpaga* spp.[†] ; *Shewanella* spp. *Arhodomonas/Saccharospirillum* [†]
5-15	100	6	11	$1.4*10^5 \pm 10^3$	$1.2*10^4 \pm 10^3$	< 5	*Raoultella* spp./*Citrobacter* spp./*Enterobacter* spp.
6-12	80	0	11	$2.6*10^5 \pm 10^4$	$9.8*10^3 \pm 10^2$	15-20	*Vibrio* spp. *; Acidovorax* spp. ; Uncultured *Bacilli Shewanella* spp. ; *Blastomonas* spp. *Acinetobacter* spp. ; *Psychrobacter* spp. *Flexibacter*/*Cytohpaga* spp. *Arhodomonas/Saccharospirillum*
Paper III (Experiment 2) and Paper IV							
8-11	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*
9-12 [δ]	*n.d.*	*n.d.*	*n.d.*	$6.6*10^4 \pm 10^3$	$5.9*10^3 \pm 10^2$	≤ 5	*Pseudoalteromonas* spp.[††]; *Pseudomonas* spp.[††] *Shewanella* spp.[†]; *Shewanella* spp.[††] *Shewanella* spp. [††] *Lacinutrix* spp./Uncultured Flavobacteriaceae[††]
5-19	47	12	2	$1.2*10^4 \pm 10^3$	$2.9*10^3 \pm 10^2$	5-10	*Vibrio*[†]
5-5 [δδ]	67	12	10	$2.1*10^4 \pm 10^3$	$1.4*10^3 \pm 10^2$	5	*Vibrio*[†] ; *Sphingobacteriales / Tenacibaculum*[††]
6-20	63	24	4	$1.3*10^4 \pm 10^2$	$0.8*10^2 \pm 10^0$	5-10	*Vibrio*[†] – *Pseudoalteromonas*[††]
6-6 [δδδ]	66	24	4	*n.d.*	*n.d.*	5-10	*Vibrio*[††]; *Pseudoalteromonas*[††]
Paper III (Experiment 3)							
8-18	26	10	0	$4.3*10^4 \pm 10^3$	$0.9*10^3 \pm 10^2$	5-10	*Vibrio*[††]; *Marinomonas*[†]; *Flexibacter*[†] *Stenotrophomonas*[†]
8-10 [δδ]	47	12	0	$6.2*10^4 \pm 10^3$	$2.8*10^3 \pm 10^2$	5-10	*Vibrio*[††]; *Acinetobacter*[†] *Sphingobacteriales/Tenacibaculum*[†]

n.d., not detected

§ colony forming units, mean values ±S.D. in duplicate to triplicate samples on marine agar (MA) and thiosulphate citrate bile salt sucrose agar (TCBS)

[†] *Products excised from the gel and identified by 16S rDNA sequences covering the variable region 4 of the gene (bp 533-787)*

[††] *Presumptively identical bands excised from parallel run samples and identified by 16S rDNA sequences covering the variable region 4 of the gene (bp 533-787)*

[δ] *Larvae originating from eggs that were bathed repeatedly in the bacterial mixture prior to hatching and offered bacteria-treated live prey in a 2*2 days treatment schedule*

[δδ] *Larvae offered bacteria-treated live prey in a 2*2 days treatment schedule*

[δδδ] *Larvae offered peptide-enriched live prey in both daily feedings throughout the first feeding period*

Higher numbers of CFU were observed in larvae after two weeks in feeding during the studies presented in ***Paper I*** compared with the studies presented in ***Papers II-IV*** (Tables 6 and 9). Analysis of bacterial numbers in larvae generally revealed between 1-2 log-unit lower numbers of CFU on TCBS compared to MA. Similar numbers of CFU were, however, observed on TCBS and MA in some of the larval samples collected during the studies presented in ***Paper I***, indicative of the overall dominance of presumptive *Vibrio* in larvae from the respective incubators (Table 9). Peptide enrichment of the live prey did not affect larval survival nor the bacterial community structure of larvae collected at various days throughout the first feeding period (***Paper IV***, Figure 5 and Table 1). Offering bacteria-treated *Artemia* to larvae, however, resulted in improved larval survival compared with the groups receiving no treatment (***Paper III***, Table 4). Larval survival varied between 48-100% during the studies presented in ***Paper I*** and between 26-67% during the studies presented in ***Papers III*** and ***IV***, with no obvious relationship to CFU numbers observed in larvae after two weeks in feeding. Variable numbers of products were furthermore observed in the DGGE gels, with highly variable groups dominating the bacterial community of larvae from different incubators (Table 9). Various products identified as *Vibrio* spp. were observed amongst the dominating bacterial community in most samples, with various groups co-dominating the bacterial community of larvae from individual incubators.

No differences in the numbers of CFU in larvae offered bacteria-treated as compared to untreated *Artemia* were observed (Table 9). A product identified as *Sphingobacteriales*/*Tenacibaculum* was, however, observed only in larvae from the group offered bacteria-treated live prey and in no samples of larvae collected from other incubators included in the studies presented in ***Papers III*** and ***IV***. Larvae originating from eggs that were bathed repeatedly in the bacterial mixture during the two week egg incubation period collapsed after a few weeks in feeding, possibly as a result of bacterial treatment during the egg stage (***Paper III***). However, untreated larvae originating from control eggs receiving no treatment also collapsed after few weeks in feeding, indicative of poor larval quality in both groups. Hence, the survival and overall success of larvae originating from treated as compared to untreated eggs could not be calculated at the end of the first feeding period (Table 9).

Overall, the results do not indicate any relationship between the bacterial community of larvae after two weeks in feeding and the overall success of larvae at the end of the first feeding period. The highly variable bacterial community observed in different samples of the live prey was only partly reflected in larvae after the first two weeks in feeding (Figure 3, ***Paper III***, Figure 2).

4.2.4 Growth and mortality of first feeding larvae

Dry weight of larvae was used for analysis of growth throughout first feeding and the numbers of CFU were calculated for the wet weight of larvae. As shown in Figure 5, the water content of larvae ranged between 83-88%, depending on larval size.

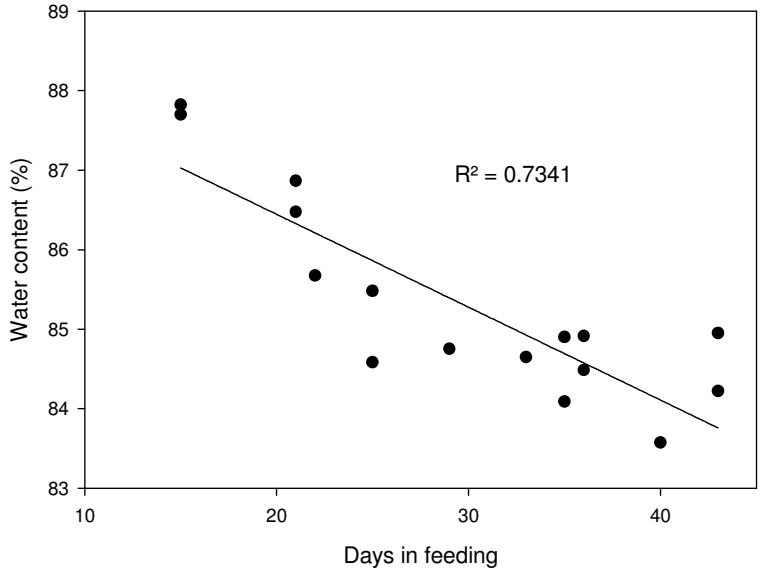

Figure 5. The water content (%) of first feeding larvae measured at various days post onset of first feeding.

Highly variable growth curves were observed for larvae from individual first feeding tanks during all periods studied. Table 10 shows the weight development of first feeding larvae in incubators resulting in the best and poorest overall survival observed during the studies presented in ***Paper I***. The last samples for evaluation of larval growth were collected between 43-53 dpff during this period and the growth curves were

therefore extrapolated to 55 dpff for comparison of the final weights of larvae from individual incubators.

Table 10. Weight and survival of first feeding larvae.
Shown is the mean weight of 10-15 larvae collected at the end of the first feeding period, from incubators resulting in the best and the poorest larval survival observed during the studies presented in Paper I. Larval weight was extrapolated to 55 days post onset of first feeding, with incubator numbering indicating the silo-tank origin of larvae.

Incubator #	Survival (%)	Weight (mg)
2-5	60 %	51.5
5-15	100 %	45.2
4-14	86 %	25.0
1-21	62 %	75.9
1-4	59 %	76.3
2-13	48 %	50.6
3-11	57 %	73.9
11-19	94 %	96.7

Extrapolation of larval growth curves to 55 dpff (Table 10) as compared to 65 dpff (***Paper I***, Figure 2), revealed no significant relationship between survival and final weights of larvae from individual incubators during the period (R^2=0.11 and 0.09, respectively), indicative of conclusive feed availability and cannibalism not representing a problem during first feeding of halibut larvae.

Similar growth curves were commonly observed for larvae of a common silo origin. Highly variable growth was, however, observed for larvae of different silo origin (***Paper I***, Figure 2). As presented in ***Paper III***, improved larval growth was observed in one of the two experiments that were carried out with bacteria-treated as compared to untreated *Artemia* offered to larvae in a sibling tank unit (***Paper III***, Experiment 2). The growth curves of larvae included in these experiments are shown in Figure 6, with "Treatment a" representing larvae that were offered bacteria-treated live prey in the two experiments.

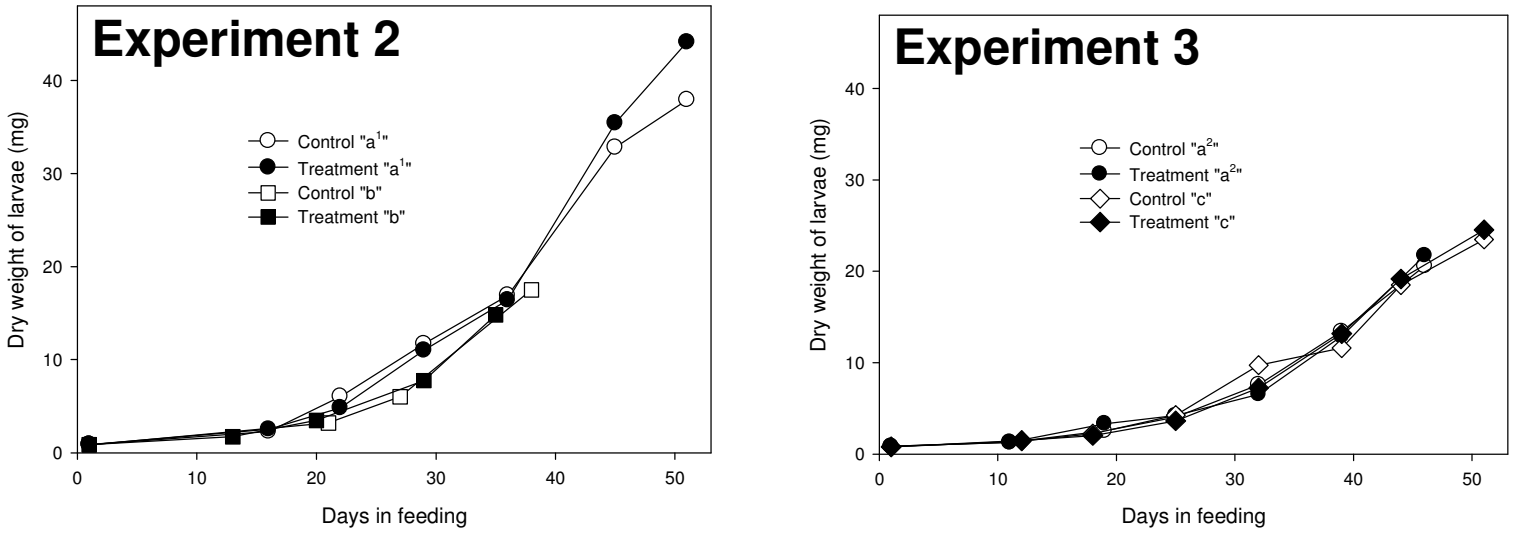

Figure 6. Growth of larvae during the studies presented in Paper III.
Shown is the weight development of untreated larvae (Control a-c) and larvae that were offered bacteria-treated live prey (Treatment "a") in the two experiments (Experiment 2 and 3) presented in Paper III. Also shown is the weight development of larvae originating from eggs that were bathed repeatedly in the bacterial mixture during the 14 days incubation of eggs (Treatment "b") or received only a single bathing immediately prior to hatching (Treatment "c") in addition to offering bacteria-treated live prey to larvae. Identical labelling (a, b, c) represents larvae of a common silo origin, with identical treatment in the two experiments denoted by a^1 and a^2.

An analysis of the CFU numbers and DGGE profiles of larvae after 55 days in feeding was not included in the studies. An analysis of the bacterial community of larvae after two weeks in feeding revealed no differences that could be related to the variable weight development and overall success of first feeding larvae (Table 9 and ***Paper III***, Table 4).

Dead larvae were enumerated in individual production units on a daily basis throughout the first feeding period in all studies. Larval mortalities in the incubators shown in Figure 6 were studied more closely, revealing variable mortality curves of larvae of different silo origin (Figure 7). During the first period studied (***Paper III***, Experiment 2), the cumulative mortality of larvae offered treated as compared to untreated live prey followed a similar pattern, with elevated larval mortalities commonly observed during the second week in feeding (Figure 7 A). In a repeated experiment carried out one year later (***Paper III***, Experiment 3), the highest larval mortalities were, however, observed after ~4 weeks in feeding in some of the incubators, including some of the incubators included in the study (Figure 7 B).

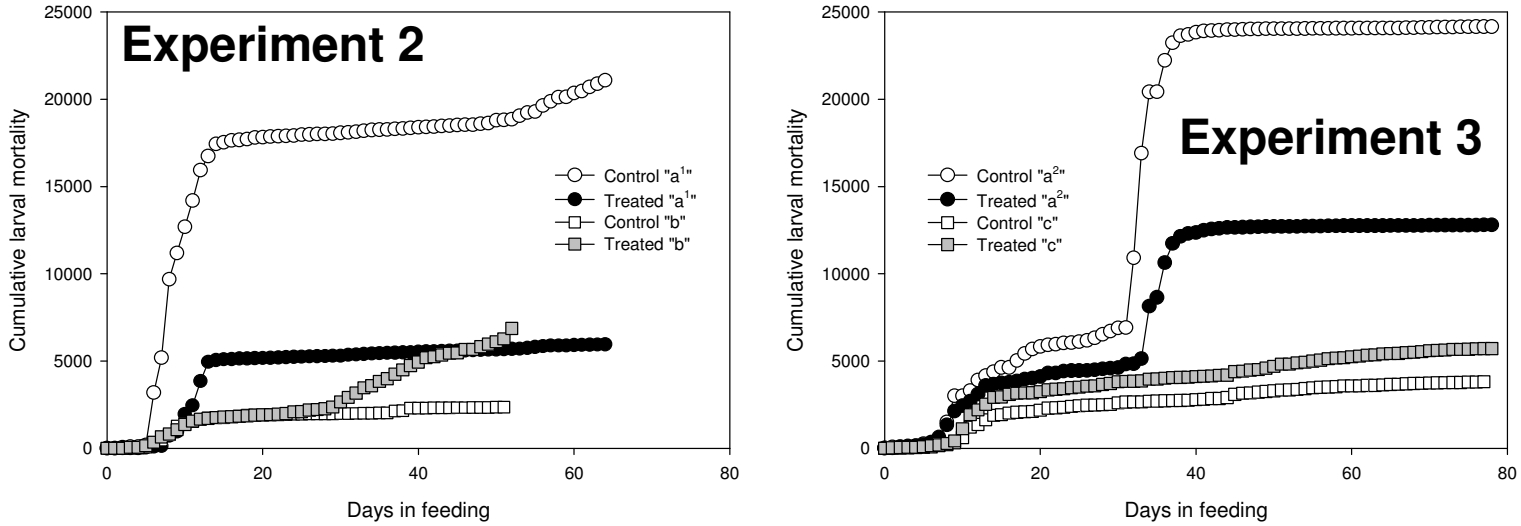

Figure 7. Cumulative larval mortality during the studies presented in Paper III.
Shown are the mortality curves of untreated larvae (Control a-c) and larvae that were offered bacteria-treated live prey (Treatment "a") in the two experiments (Experiment 2 and 3) presented in Paper III. Also shown is the cumulative mortality of larvae originating from eggs that were bathed repeatedly in the bacterial mixture during the 14 days incubation of eggs (Treatment "b") or received only a single bathing immediately prior to hatching (Treatment "c") in addition to offering bacteria-treated live prey to larvae. Identical labelling (a, b, c) represents larvae of a common silo origin, with identical treatment in the two experiments denoted by a^1 and a^2.

Analysis of the bacterial community of larvae after one week in feeding, i.e. during the period of increased larval mortalities shown in Figure 7, revealed a product identified only in larvae from the control groups shown in Figure 7 (Control a). Excising and subsequent sequence analysis identified a *Vibrio* spp. as the closest relative (GeneBank accession number AM941184.1) (***Paper III***, Figure 2 product # 18). An identical product was furthermore observed in samples of the live prey collected during the period (***Paper III***, Figure 2 product # 30).

The product may no longer be observed in larvae after three weeks in feeding (***Paper III***, Figure 3), when only low mortalities were observed (Figure 7 A). In a repeated experiment, a product appearing at the same location may furthermore be observed in both treated and untreated larvae collected after three weeks in feeding (***Paper III***, Figure 3 unlabelled product), i.e. prior to the increased larval mortalities observed after ~4 weeks in feeding (Figure 7 B). The results therefore indicate the presence of this particular *Vibrio* spp. isolate in relation to increased larval mortalities during the studies presented in ***Paper III***.

The ratio of surviving larvae was found to vary significantly when individual production units were compared during the studies presented in ***Paper I*** (***Paper I***, Figure 1). A closer analysis of sibling tank units containing larvae of a common silo origin revealed no relationship between the survival of unfed yolk sac larvae and the corresponding first feeding larvae (R^2=0.02) during the period (***Paper I***, Figure 1). A negative relationship was, however, observed between the incidence of gaping amongst unfed yolk sac larvae and survival of the corresponding group of first feeding larvae (R^2=0.53). The most successful first feeding larvae during the period originated from a yolk sac incubator resulting in average larval survival but the highest percentage of gaping observed during the period. In agreement with the results shown in Figure 7A, the mortality curves of larvae from the most and least successful first feeding incubators during the studies presented in ***Paper I*** revealed elevated mortality in all tanks after 5-12 days of feeding (Figure 8).

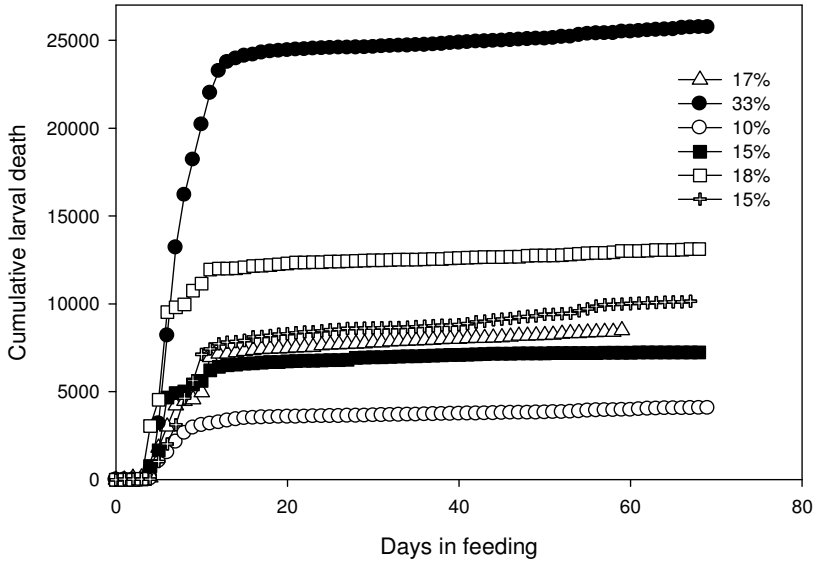

Figure 8. Cumulative larval mortality during the studies presented in Paper I.
Shown are larval mortalities in incubators resulting in the highest and the poorest larval survival observed during the period, with larvae from sibling tank units denoted with identical symbol forms. Symbol labelling in % indicates the ratio of incomplete metamorphoses observed amongst the surviving larvae.

No relationship was observed between the numbers of CFU in larvae collected at various dpff and larval survival at the end of the studies presented in ***Paper I*** (***Paper I***, Figure 1 and Table 1). The DGGE profiles of larvae after one week in feeding, i.e. during a period with increased larval mortalities observed in all incubators, revealed no products that might be related to the elevated larval mortalities observed in the individual incubators (***Paper 1***, Figure 5).

4.2.5 Immune stimulation of first feeding larvae

The effects of life feed enrichment using a fish peptide hydrolysate, are presented in ***Paper IV***. Briefly, the immune parameters studied, IgM, C3 and lysozyme, were detected in various organs and cellular layers of the digestive system of larvae already at the onset of feeding, however, with considerable differences in the distribution and

magnitude observed when individual larvae were compared (***Paper IV***, Figures 2-4). Higher individual variability in the presence of IgM was observed in untreated larvae as compared to larvae offered peptide-enriched life feed. However, an analysis of the concentration of IgM in larvae collected throughout the first feeding period revealed no significant difference between the two groups (***Paper IV***, Figure 1). Already after the first week in feeding, C3 had a clearly more prominent and widespread distribution in the digestive system of treated compared with untreated larvae. A considerable individual variability in the distribution of lysozyme was furthermore observed in larvae from the untreated as compared with the treated group, with a distinct positive response observed in treated larvae after seven weeks in feeding (***Paper IV***, Figure 4). Overall, the results indicate a stimulation of selected immune parameters as a result of offering peptide-enriched live prey to larvae, however, without affecting larval survival or the bacterial community structure of larvae (***Paper IV***, Figure 5). The selected treatment schedule may, however, have negatively affected the normal development of larvae, whereas a lower ratio of treated larvae developed to successfully metamorphosed fry.

4.3 Culture water

4.3.1 Seawater for production

Figure 9 shows the numbers of CFU in seawater used for production during the studies presented in ***Paper II*** (2001-2003). Only low numbers of CFU were observed on both MA (10^2–10^3 CFU mL^{-1}) and TCBS ($\leq$10 mL^{-1}), with an increase in numbers of CFU on TCBS commonly observed during late autumn (Figure 9).

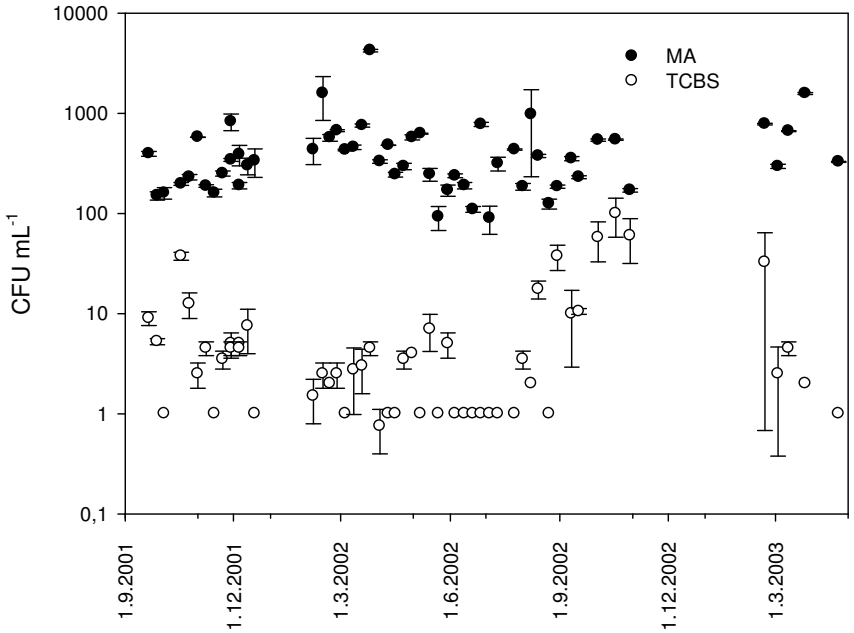

Figure 9. Seawater for production.
Shown are numbers of colony forming units (CFU) in each mL of seawater collected during the studies presented in Paper II. Total numbers of CFU were determined on marine agar (MA) and presumptive *Vibrio* bacteria on thiosulphate citrate bile salt sucrose agar (TCBS).

4.3.2 Incubation of halibut eggs

Analysis of the bacterial community of fertilized eggs is presented in ***Paper III*** (***Paper III***, Figure 1). The DGGE profiles of the culture water from some of the incubators included in the study are shown in Figure 10.

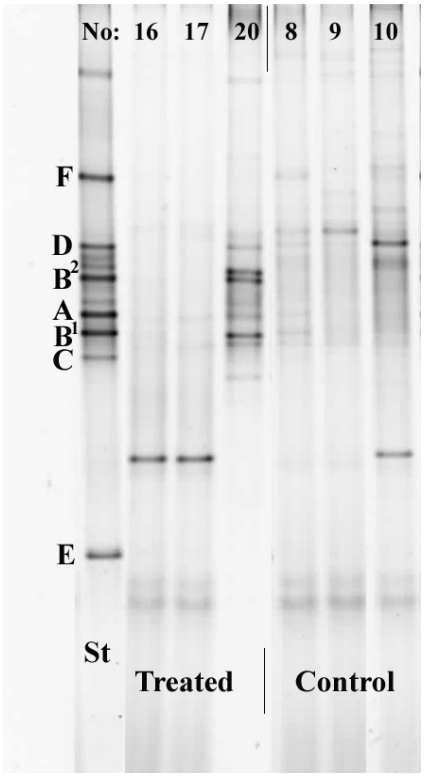

Figure 10. DGGE profiles of the culture water of eggs.
Shown are the profiles of eggs from three incubators containing untreated eggs (Control) and three incubators containing eggs that were bathed repeatedly in a mixture of the bacterial isolates (Treated). The samples were collected one day after treatment, seven days post fertilization, with labelling (No) indicating the incubator origin of eggs. Also shown are relative mobility standards (St), represented by the *Pseudoaltermonas elyakovii* (A), *Vibrio* spp. (B^1) and *V. splendidus* (B^2) isolates used for bacterial treatment in addition to *Marinovum algicola* (C), *Shewanella baltica* (D), *Streptomyces* spp. (E) and *Staphylococcus aureus* (F). The gel contains 30-60% urea-formamide denaturing gradient and 10% acrylamide-bis. The bacteria are represented by 16S rDNA sequences covering the variable region 4 of the gene (bp 358-517).

Overall, the DGGE profiles of the culture water (Figure 10) showed less diversity of products compared to the profiles observed in eggs (***Paper III***, Figure 1). Products were not excised from the gel for identification by sequence analysis. Products identical to the bacterial isolates used for treatment (products A and B in the relative mobility standard) were detected in treated eggs only and in no samples of eggs collected from the control incubators (***Paper III***, Figure 1 and Table 5). As shown in Figure 10, all three isolates may be observed in the culture water of treated eggs from one of the incubators included in the study (incubator No. 20 in Figure 10) but in none of the control incubators included in the study.

4.3.3 The culture water during production of halibut larvae

The bacterial community in the culture water of first feeding larvae was analysed only in the studies presented in ***Paper II***, with fermentative Gram negative bacteria dominating the cultivable bacterial community in the tank water of larvae from both groups (***Paper II***, Figure 1). After two weeks of feeding, significantly higher numbers of CFU were found in the tank water of larvae with environmental shading provided by marine microalgae as compared to inorganic clay ***(Paper II***, Figure 1). The studies presented in ***Paper II*** were carried out during 1999-2002. The use of marine microalgae as compared to inorganic clay for environmental shading during first feeding was studied further in March 2009, in sibling tank units containing larvae of a common silo origin. The numbers of CFU in the culture water of larvae during the two studies are summarized in Table 11. Similar numbers of CFU on MA were observed in the culture water of larvae with environmental shading provided by marine microalgae and inorganic clay during the two studies. However, approximately one log-unit lower numbers of CFU were observed on TCBS with environmental shading provided by inorganic clay as compared to marine microalgae during both studies (Table 11).

Table 11. Bacterial numbers in the culture water of first feeding larvae.
Shown are the numbers of colony forming units (CFU) on MA and TCBS after 7 and 14 days of feeding (dpff), with environmental shading provided by inorganic clay or marine microalgae. CFU numbers during the studies presented in Paper II show mean values ±S.D. in duplicate samples collected from ≥7 incubators from each group, while the CFU numbers during the studies carried out in 2009 show mean values ±S.D. in duplicate samples collected from sibling tank units containing larvae of a common silo origin.

	Marine microalgae				**Inorganic clay**			
	7 dpff		**14 dpff**		**7 dpff**		**14 dpff**	
	CFU (MA) [§]	**CFU (TCBS)**	**CFU (MA)** [§]	**CFU (TCBS)** [§]	**CFU (MA)** [§]	**CFU (TCBS)**	**CFU (MA)** [§]	**CFU (TCBS)**
Paper II	$3.5*10^5 \pm 10^5$	$1.9*10^3 \pm 10^3$	$1.1*10^6 \pm 10^5$	$4.9*10^3 \pm 10^3$	$1.1*10^5 \pm 10^5$	$1.0*10^3 \pm 10^3$	$1.0*10^5 \pm 10^5$	$2.4*10^2 \pm 10^2$
2009	$3.7*10^5 \pm 10^5$	$4.0*10^4 \pm 10^4$	$1.2*10^6 \pm 10^4$	$4.5*10^3 \pm 10^3$	$1.1*10^5 \pm 10^4$	$0.3*10^3 \pm 10^2$	$0.5*10^5 \pm 10^4$	$5.5*10^2 \pm 10^2$

§ Colony forming units (CFU), mean values ±S.D. in duplicate samples on marine agar (MA) and thiosulphate citrate bile salt sucrose agar (TCBS)

Analysis of the culture water of larvae during the study carried out during 2009 (Table 8) revealed similar DGGE profiles for the two groups (Figure 11). Products identified as *Marinomonas* spp. and *Shewanella* spp. (Figure 11, products 5 and 6), however, were observed only in culture water with environmental shading provided by marine microalgae (Table 12). A product identified as *Polaribacter*/Flavobacteriaceae (Figure 11, product 1) may furthermore be observed only with environmental shading provided by inorganic clay. The overall results indicate different DGGE profiles, however, with similar groups identified in the culture water and larvae from the respective incubators (Figure 3 and 11, Table 7 and 12). Various products that were identified as *Vibrio* spp. and *Pseudoaltermonas* spp. in addition to *Marinomonas/ Oceanospirillales, Thalassomonas, Altermonas/Glaciecola* and *Psychrobacter* dominated the bacterial community in the culture water of larvae in both groups.

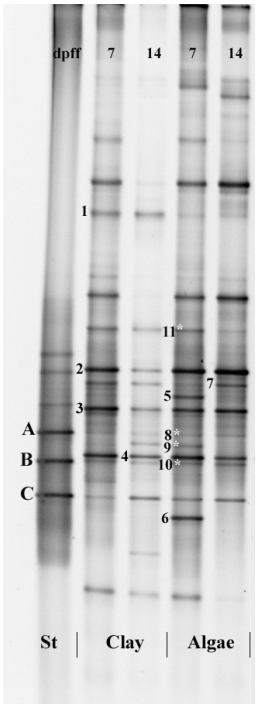

Figure 11. DGGE profiles of the culture water of first feeding larvae.
Shown are the profiles in the culture water of larvae at 7 and 14 days post onset of first feeding (dpff), in incubators containing first feeding larvae of a common silo origin and with environmental shading provided by inorganic clay (Clay) or marine microalgae (Algae). Also shown are relative mobility standards (St.), represented by *Pseudoalteromonas elyakovii* (A), *Vibrio splendidus* and *Vibrio* spp. (B) and *Marinovum algicola* (C). Labelled products indicate bands that were excised from the gel and identified by sequence analysis (Table 12). Products labelled with an asterisk (*) indicate presumptively identical bands excised from parallel run samples that were identified by sequence analysis. The gel contains 30-60% urea-formamide denaturing gradient and 8% acrylamide-bis. The bacteria are represented by 16S rDNA sequences covering the variable region 4 of the gene (bp 533-787).

Table 12. Identification of products excised from the gel shown in Figure 11.
Groups identified as part of the bacterial community of the culture water of first feeding halibut larvae in tanks with environmental shading provided by inorganic clay as compared to marine microalgae. Products labelled with an asterisk (*) indicate presumptively identical bands excised from parallel run samples. The products identified are represented by 16S rDNA sequence covering the variable region 4 of the gene (bp 533-787).

Product	BLAST identification	Division (% similarity)	GeneBank link
1	Flavobacteriaceae	Flavobacteria (96%)	EU394573
	Polaribacter glomeratus strain	Flavobacteria (96%)	EU000227
	Uncultured bacterium	(96%)	AM921603
2	*Marinomonas* spp.	γ-Proteobacteria (95%)	FJ457290
	Oceanospirillales bacterium	γ-Proteobacteria (95%)	DQ810540
	Uncultured marine bacterium	(95%)	EU338370
3	Uncultured bacterium PENDANT-8	γ-Proteobacteria (93%)	AF142921
	Thalassomonas haliotis	γ-Proteobacteria (92%)	AB369381
4	*Glaciecola* spp.	γ-Proteobacteria (96%)	EU268077
	Alteromonas spp.	γ-Proteobacteria (96%)	EU600749
5	*Marinomonas* spp.	γ-Proteobacteria (99%)	AF277471
6	*Shewanella* spp.	γ-Proteobacteria (97%)	FJ626842
7	*Marinomonas* spp.	γ-Proteobacteria (94%)	FJ457290
	Oceanospirillales bacterium	γ-Proteobacteria (94%)	DQ810540
8*	*Pseudoalteromonas* spp.	γ-Proteobacteria (98%)	FJ404756
9*	*Vibrio* spp.	γ-Proteobacteria (96%)	AM989925
	Vibrio vulnificus	γ-Proteobacteria (96%)	EF546308
10*	*Vibrio* spp.	γ-Proteobacteria (100%)	FJ596492
	Vibrio splendidus	γ-Proteobacteria (100%)	FM954973
11*	*Psychrobacter* spp.	γ-Proteobacteria (100%)	FJ039851

4.4 The live prey offered to larvae

An analysis of the bacterial community in samples of the live prey is presented in ***Papers I***, ***III*** and ***IV***. Overall, highly variable numbers of CFU were observed in samples of the live prey offered to larvae, also in samples collected within the individual periods studied. The numbers of CFU were between $8.4*10^3$ to $4.1*10^6$ g^{-1} *Artemia* (wet weight) during the studies presented in ***Paper I***, with presumptive *Vibrio* bacteria dominating the cultivable bacterial community in all samples ($1.0*10^2$ to $1.1*10^4$ CFU on TCBS g^{-1} *Artemia*). Grazing of the live prey in seawater containing a fish peptide hydrolysate or a mixture of selected bacterial isolates did not affect the numbers of CFU observed in the *Artemia* (***Papers III*** and *IV*). A regression analysis, however, revealed a positive relationship between the numbers of CFU on MA and TCBS only in samples of untreated *Artemia* and not in bacteria-treated *Artemia* (***Paper III***). Highly variable DGGE profiles were furthermore observed in samples of the live prey and various groups were identified in samples collected within the individual periods studied (***Paper III***, Figure 2 and ***Paper I***, Figures 5-7). In summary, the results indicate an overall dominance of various products identified as *Vibrio* spp., with *Pseudomonas* spp., *Pseudoalteromonas* spp. *Corynebacterium* spp. *Acinetobacter* spp., *Moraxella* spp. and *Tenacibaculum* spp. identified amongst the dominating bacterial community in individual samples of the live prey.

Analysis of the bacterial community of newly hatched *Artemia* cysts revealed variable DGGE profiles in individual animals (Figure 12). Excising and subsequent sequence analysis of selected products revealed the identification of various products that were identified as *Vibrio* spp. in most samples (Table 13). Products identified as *Stenotrophomonas* spp., *Hydrogenophaga* spp. and *Bacillus/Anoxybacillus/Tepidomonas* were furthermore found to co-dominate the bacterial community in individual animals.

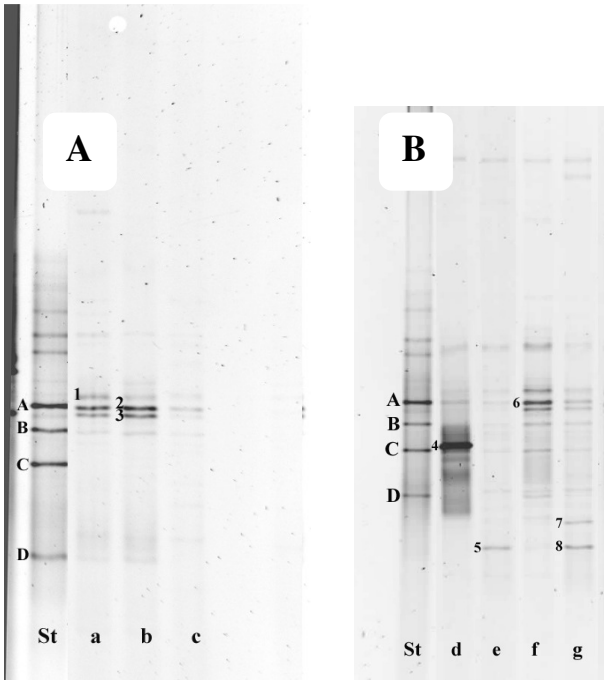

Figure 12. DGGE profiles of newly hatched *Artemia* cysts.
Shown are the profiles in 7 samples (a-g), each consisting of a single animal and run in separate gels (A, B). Also shown are relative mobility standards (St.), represented by *Pseudoalteromonas elyakovii* (A), *Vibrio splendidus* and *Vibrio* spp. (B), *Marinovum algicola* (C) and *Shewanella baltica* (D). Labelled products indicate bands that were excised from the gel and identified by sequence analysis (Table 1). The gel contains 30-60% urea-formamide denaturing gradient and 8% acrylamide-bis. The bacteria are represented by 16S rDNA sequences covering the variable region 4 of the gene (bp 533-787).

Table 13. Identification of products excised from the gels shown in Figure 12.
Groups identified as a part of the bacterial community of individual newly hatched *Artemia* cysts, run in two separate gels (A, B). The products identified are represented by 16S rDNA sequence covering the variable region 4 of the gene (bp 533-787).

Labelling Figure 12:	Product no.	BLAST identification	Division (% similarity)	GeneBank accession no.
A	1	Uncultured bacterium clone	93%	EU438296
	2	*Vibrio* spp.	γ-Proteobacteria (99%)	FJ178079
	3	*Vibrio* spp.	γ-Proteobacteria (99%)	FJ178084
B	4	*Stenotrophomonas* spp.	γ-Proteobacteria (98%)	EU054384
	5	*Hydrogenophaga* spp.	β-Proteobacteria (97%)	DQ854970
	6	*Vibrio* spp.	γ-Proteobacteria (92%)	AM989925
	7	*Bacillus* spp.	Bacillaceae (95%)	FJ215800
		Anoxybacillus spp.	Bacillaceae (95%)	AM999779
	8	Unculture *Tepidimonas* spp.	β-Proteobacteria (95%)	EF648100
		Hydrogenophaga spp.	β-Proteobacteria (94%)	DQ854970

The bacterial community of *Artemia* during different developmental stages was furthermore studied more closely. Similar numbers of CFU were observed in decapsulated and newly hatched *Artemia* cysts, with 0.8-2.0*10^2 ±10^2 CFU g^{-1} observed on MA and 0.3-0.8*10^2 ±10^2 CFU g^{-1} on TCBS (mean values ±S.D. from the analysis of four samples). Enrichment of the *Artemia* for 24 h may have resulted in increased numbers of CFU, with 3*10^3 ±10^3 CFU g^{-1} observed on MA and 0.1-0.2*10^3 ±10^2 CFU g^{-1} on TCBS. No differences in CFU numbers were observed when decapsulated *Artemia* cysts from different producers were compared (results not shown). The DGGE profiles of the samples, however, indicated a somewhat different bacterial community structure of *Artemia* cysts obtained from different producers (Figure 13). A change in the bacterial community structure was furthermore observed following *Artemia* culturing, with products dominating the bacterial community of cysts no longer observed as a part of the community structure following hatching of the *Artemia* cysts (Figure 13). Similar DGGE profiles, however, were observed for newly hatched *Artemia* cysts and following enrichment of the *Artemia* for 24 h. Products were not excised from the gels for identification by sequence analysis.

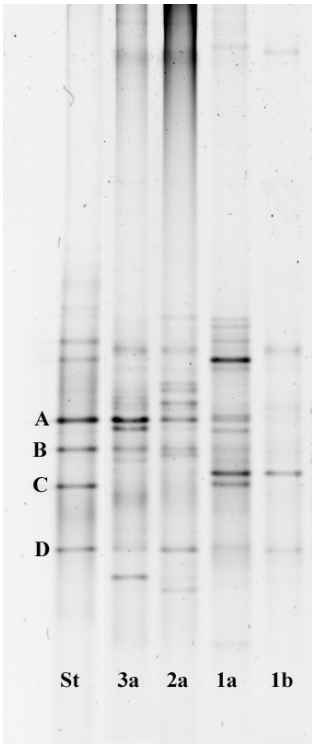

Figure 13. DGGE profiles of *Artemia* during various developmental stages.
Shown are the profiles in pools of *Artemia* cysts (1a, 1b), newly hatched *Artemia* cysts (2a) and *Artemia* after enrichment for 24 h (3a). The live prey originated from two different producers (a, b). Also shown are relative mobility standards (St.), represented by *Pseudoalteromonas elyakovii* (A), *Vibrio splendidus* and *Vibrio* spp. (B), *Marinovum algicola* (C) and *Shewanella baltica* (D). The gel contains 30-60% urea-formamide denaturing gradient and 8% acrylamide-bis.The bacteria are represented by 16S rDNA sequences covering the variable region 4 of the gene (bp 533-787).

5 DISCUSSION

The present studies describe analysis of the bacterial community structure at various stages during intensive production of halibut larvae. The bacterial community of larvae and their culture water were furthermore studied with environmental shading provided by marine microalgae as compared to inorganic clay. Also studied were the effects of treatment using a mixture of three autochthonous bacteria or a pollock protein hydrolysate on the bacterial community and larval development. Enumeration of cultivable bacteria in seawater used for the production and an analysis of the bacterial community structure at various developmental stages of the live prey was also included in the studies.

5.1 Cultivable bacterial numbers (Papers I-IV)

In agreement with previous observations (Jensen *et al.*, 2004a; Olsen *et al.*, 2000; Tolomei *et al.*, 2004; Verner-Jeffreys *et al.*, 2003b), *Vibrio* spp. dominated the bacterial community of first feeding larvae and their live prey. A satisfactory recovery of *Vibrio* spp. was furthermore obtained using the TCBS medium, a semi-selective nutrient media commonly used for enumeration of presumptive *Vibrio* bacteria (Olsen *et al.*, 2000; Tolomei *et al.*, 2004). Recovery of bacteria from the marine environment has, however, proven difficult by culturing methods and the cultivable part of the bacterial community has been found to represent only a small part of the microbial diversity in environmental samples (Hongoh *et al.*, 2003; Keller and Zengler, 2004; Olafsen, 2001). In agreement with previously published observations (Nakase and Eguchi, 2007; Tolomei *et al.*, 2004; Verner-Jeffreys *et al.*, 2006), the present results, however, indicated that groups dominating the bacterial community might to a large extent be recoverable on traditional nutrient media.

The variable numbers of CFU observed in bacteria-treated and untreated eggs revealed no differences that could be related to the highly variable survival rates of eggs observed in individual incubators included in the study (***Paper III***). In agreement with previously published studies (Jensen *et al.*, 2004a; Verner-Jeffreys *et al.*, 2003b), CFU numbers below $5*10^5$ larvae^{-1} were generally observed in larvae prior to the onset of

exogenous feeding. Similar numbers of CFU were observed in unfed yolk sac larvae during the individual periods studied, but highly variable numbers were observed during the different periods studied, indicative of an overall variable quality of unfed yolk sac larvae during different periods. A positive relationship was commonly observed between the numbers of CFU larvae^{-1} and the prevalence of gaping amongst unfed yolk sac larvae, a jaw deformation that has been associated with abrasion of the surface layers and subsequent invasion by microorganisms (Ottesen and Olafsen, 2000). The highest numbers of CFU were, however, found in yolk sac larvae from the most successful production unit during the studies presented in ***Paper I***, indicative of the establishment of a favourable bacterial community. Overall, the results therefore support previous suggestions of complex interactions of bacterial communities in the rearing system (Olafsen, 2001).

A considerable increase in CFU numbers was observed in the larval gut during the first days in feeding, however, without the previously observed increase in CFU numbers observed as a result of grazing of the live prey in a mixture of bacteria prior to offering to larvae (Makridis *et al.*, 2000a). As discussed in ***Paper I***, a positive relationship was observed between the CFU numbers in unfed yolk sac larvae and poor quality of the respective first feeding larvae measured as incomplete eye migration, a commonly observed defect suggested to be caused by environmental and nutritional factors (Hamre and Harboe, 2008b; Harboe *et al.*, 2009; Solbakken and Pittman, 2004).

The highest numbers of CFU in the culture water of first feeding larvae were observed after approximately two weeks of feeding, coinciding with the period of a sudden larval death commonly observed. Significantly higher CFU numbers were furthermore observed in the culture water with environmental shading provided by marine algae as compared to inorganic clay. Marine microalgae have been considered essential for normal development of marine fish larvae, as nutritional supplement as well as for the effects on physical parameters such as the light regime (Naas *et al.*, 1992; Palmer *et al.*, 2007; Rocha *et al.*, 2008; van der Meeren *et al.*, 2007). Using marine microalgae, however, represents an increase in the nutrient availability within the production system that has been suggested to support the multiplication of opportunistic bacteria (Hjelm *et al.*, 2004b; Makridis *et al.*, 2006; Nakase and Eguchi, 2007; Olafsen,

2001; Pinhassi *et al.*, 2004). Increased nutrient availability may therefore be expected to result in a more unstable environment, whereas stable environmental conditions have been suggested to prevent an unpredictable development of bacterial communities (Olsen *et al.*, 2000). As pointed out by numerous authors, however, marine microalgae may positively affect the bacterial community of the live prey and the intestinal flora of fish larvae as bacterial selecting factor (Austin *et al.*, 2006; Liao *et al.*, 2003; Makridis *et al.*, 2006; Marques *et al.*, 2006; Olsen *et al.*, 2000). As discussed in ***Paper II***, substituting the algae for inorganic clay represented a considerable reduction in the production costs and the overall production safety, and inorganic clay has since 2003 been used exclusively during first feeding of larvae at the commercial production site at Fiskey Ltd.

In agreement with previous studies (Olsen *et al.*, 2000; Verner-Jeffreys *et al.*, 2003b), members of the *Vibrio* group dominated the cultivable bacterial community of the live prey. Highly variable CFU numbers were observed in various samples of the live prey, but relatively lower compared to previously published studies (López-Torres and Lizárraga-Partida, 2001; Savas *et al.*, 2005). In agreement with previous findings (Høj *et al.*, 2009), higher numbers of CFU and presumptive *Vibrio* bacteria were observed following live prey enrichment as compared to newly hatched *Artemia* cysts.

Variable percentages of isolates dominating the cultivable bacterial community showed a poor response in the morpho-physiological tests applied or did not grow upon subculturing. This was more commonly observed when analysing the culture water as compared to larvae and may have reflected nutrient richness that have been suggested to contribute to the success of cultivation of bacteria from the marine environment (Kemp and Aller, 2004; Tolomei *et al.*, 2004). Monitoring of the environment furthermore involves detecting specific bacteria in low concentrations against a large background of a variety of bacterial groups. The cultivability of only a part of the total bacterial community in environmental samples therefore suggested that the total community structure should be considered rather than bacterial numbers, as previously pointed out by other authors (Makridis *et al.*, 2000b; Zhou *et al.*, 2009).

5.2 Analysis of the bacterial community using PCR-DGGE (Papers I, III, IV)

5.2.1 Methodology

Different phylotypes of bacteria in samples of larvae, their culture water and live prey were assessed using PCR and the DGGE method. This method has commonly been applied for fingerprinting of bacterial populations in aquaculture and, as in the present studies, often combined with traditional culturing on non-selective nutrient media (Brunvold *et al.*, 2007; Dorigo *et al.*, 2005; Griffiths *et al.*, 2001; Hovda *et al.*, 2007; Huber *et al.*, 2004; Jensen *et al.*, 2004a).

The 341F-GC and 534R bacterial universal primers initially tested during the method development have frequently been selected for analysing total community structures (Watanabe *et al.*, 2001). Running the amplified DNA in DGGE using 30-60% urea-formamide denaturing agent and 10% acrylamide, however, resulted in the co-amplification of eukaryotic 18S rDNA from the larvae and their live prey. Co-amplification of eukaryotic DNA has also been experienced when using the 338F-GC and 518R primers that are also directed against the V3 region of the 16S gene (Jensen *et al.*, 2004a). Only the most dominating bacterial groups will become visible in the gels and the co-amplification of eukaryotic DNA from the larvae and the live prey, dominating in all samples, may therefore be expected to exclude bacterial groups present in lower relative quantities. This may explain the few products observed in the gels when using the 341F-GC and 534R primers during our method development (results not shown).

In agreement with the observations of Sekiguchi *et al.* (2001), more than one group was occasionally identified in products excised from the gels. As previously pointed out by Bodelier *et al.* (2005), only a small part of the 16S rDNA is analysed using the DGGE method and whereas different bacterial groups may contain a similar base-pair composition within the particular region of the gene, albeit in a different sequence, the fragments will resolve at the same location within the gel. New gels must furthermore be made for every run and these will never be exactly identical. Hence, it is important to take into account the gradient of the gel when comparing samples run in different gels, as was done in the present study. The use of relative mobility standards

will counterbalance this potential problem, and it is therefore important to use adequate standards that range the gradient of the gel.

5.2.2 Halibut eggs and unfed yolk sac larvae

The variable DGGE profiles observed in eggs and their culture water revealed no differences that could be related to the highly variable survival of eggs observed in individual incubators included in the study (***Paper III***). Our results are therefore in agreement with previous observations indicating that the microflora of eggs may vary both quantitatively and qualitatively (Hansen and Olafsen, 1999; Olafsen, 2001; Verner-Jeffreys *et al.*, 2006). *Tenacibaculum ovolyticum* dominated the bacterial community of eggs together with *Pseudoalteromonas/Lacinutrix* and *Marinomonas*. Products identical to the isolates selected for bacterial manipulation were detected as a part of the dominating bacterial community of treated eggs but not in eggs from any of the control incubators, indicative of a successful colonization of the eggs (***Paper III***). Egg survival was not affected by the treatment and a successful colonization of the putative probionts might therefore have provided protection against opportunistic colonization by bacteria, as previously suggested (Høj *et al.*, 2009; Olafsen, 2001). Whereas the intestinal bacterial community of marine larvae is established by the ingestion of bacteria by drinking long before the larvae actually start feeding (Olafsen, 2001), creating a hostile environment for opportunistic pathogens prior to hatching may be important for improved overall production performances.

In agreement with the observations of Verner-Jeffreys *et al*. (2003b), highly variable DGGE profiles were observed in unfed yolk sac larvae collected from different production units included in the studies. Also in agreement with previous findings (Jensen *et al.*, 2004a), *Marinomonas* spp. was identified as a part of the dominating bacterial community of larvae from most incubators during the studies presented in ***Paper I***, but not in larvae from any of the incubators included in the studies presented in ***Papers III*** and ***IV***. As previously observed (Verner-Jeffreys *et al.*, 2003b), *Vibrio* spp. were commonly found amongst the dominating bacterial community of unfed yolk sac larvae during some periods studied (***Papers III*** and ***IV***), but only sporadically during

other periods (***Paper I***). Overall, the results indicate the dominance of *Marinomonas, Marinobacter* and *Pseudoalteromonas* in addition to *Stenotrophomonas*, with various other groups colonizing unfed yolk sac larvae during the different periods studied. In general, the DGGE profiles of unfed yolk sac larvae revealed a higher diversity compared to the results of Jensen *et* al. (2004a), who studied the bacterial community of unfed yolk sac larvae from the hatchery at Fiskey Ltd. and that were collected immediately prior to the onset of feeding, as in the present studies. This may be partly explained by the different set of primers and DGGE methodology applied. Also, the co-amplification of 18S rDNA in the study of Jensen *et al*. (2004) may, due to the overall dominance of eukaryotic DNA in all samples, be expected to exclude bacterial groups present in lower relative quantities. Those authors identified *Marinomonas* and *Pseudomonas* as colonisers of yolk sac halibut larvae, together with *Janthinobacterium* that were not identified as a part of the bacterial community of yolk sac larvae in the present studies. This group may, however, be present as β-Proteobacterium*/Citrobacter* that was indentified as a dominating product in the most successful larvae during the studies presented in ***Paper I***. Members of the *Pseudoalteromonas* group produce a range of biologically active compounds and the group has been suggested to provide protection to unfed yolk sac larvae (Verner-Jeffreys *et al.*, 2004). In agreement with previous findings (Verner-Jeffreys *et al.*, 2003b), members of the *Pseudoalteromonas* group frequently dominated the intestinal bacterial community of unfed yolk sac larvae in the present studies, but also in larvae from incubators resulting in overall poor survival.

5.2.3 First feeding larvae

Vibrio spp. dominated the bacterial community of surface sterilized first feeding larvae, as previously observed by other authors (Jensen *et al.*, 2004b; Verner-Jeffreys *et al.*, 2003b). *Shewanella*, *Pseudomonas*, *Acidovorax* and *Psychrobacter* were among groups that co-dominated the bacterial community of larvae. Other authors have furthermore identified *Pseudoalteromonas*, *Photobacterium* and *Marinmonas* as a part of the dominating bacterial community of first feeding halibut larvae (Jensen *et al.*, 2004b) and

these groups, together with *Pseudomonas* and *Marinobacter*, were identified only after the first week in feeding, indicative of the yolk sac origin of the respective groups. A product identified as *Marinomonas* was furthermore detected only in larvae from incubators with environmental shading provided by marine microalgae and not by inorganic clay.

The α-Proteobacterium group was identified in first feeding larvae during the studies presented in ***Paper I***. Members of the group have commonly been identified in the marine environment (Schafer *et al.*, 2002; Selje *et al.*, 2004) but have not previously been reported in halibut larvae (Jensen *et al.*, 2004a). Members of the *Roseobacter* clade, belonging to this group, have been observed in high densities in rearing environments of marine larvae, with various strains identified as putative probionts for marine larvae (Hjelm *et al.*, 2004b; Planas *et al.*, 2006; Ruiz-Ponte *et al.*, 1999; Vine *et al.*, 2006). However, α-Proteobacterium was not identified in larvae during the studies presented in ***Papers III*** and ***IV***, and the lack of dominance in larvae from some of the incubators included in the studies presented in ***Paper I*** may indicate the presence of this group only in lower proportions during intensive production of halibut larvae, or with seasonal incidence, as has been suggested (Hjelm *et al.*, 2004b). Another member of the *α*-Proteobacterium group, *Stenotrophomonas* spp. was occasionally observed in feeding larvae during the studies presented in ***Paper I***, and sporadically during the studies presented in ***Paper III***, however, without any relationship to larval survival observed. The predominance of α–Proteobacteria and the *Cytophaga – Flavobacterium* cluster has been found to be a good indicator for successful production of, for example, sea bream larvae (Nakase *et al.*, 2007). Both groups were identified as a part of the bacterial community of halibut larvae, however, without any relationship with larval survival observed.

Bioencapsulation of the live prey using a fish peptide hydrolysate did not affect the bacterial community structure or survival of first feeding larvae (***Paper IV***). Grazing of the live prey in a mixture of bacterial isolates dominating the cultivable gut community of overall successful larvae, however, resulted in improved larval survival (***Paper III***). The three isolates selected for bacterial treatment, *Vibrio* spp., *V. splendidus* and *Pseudoalteromonas elyakovii,* all belong to genera commonly isolated from fish and

their environment and that have been tested as probionts in cultures of fish (Fjellheim *et al.*, 2007; Gomez-Gil *et al.*, 2000; Hjelm *et al.*, 2004a; Makridis *et al.*, 2005). As discussed in detail in ***Paper III***, *V. splendidus* has been implicated as a potential pathogen for e.g. turbot larvae (Gatesoupe *et al.*, 1999; Thomson *et al.*, 2005). The relative dominance of this group within the intestinal community of healthy and overall successful halibut larvae and the improved larval survival obtained as a result of the treatment may, however, implicate the lack of a definition of the different organisms belonging to this group, as previously suggested (Gatesoupe *et al.*, 1999; Verner-Jeffreys *et al.*, 2003a). In agreement with previous findings (Gatesoupe, 2002; Gomez-Gil *et al.*, 2000; Suzer *et al.*, 2008), bacterial bioencapsulation of the live prey furthermore resulted in improved larval growth in one of the two experiments that were carried out. Improved growth during early production stages has been found to persist during the on-growing phase (Imsland *et al.*, 2007), stressing the importance of a healthy gut community of larvae during early production stages. Previous findings indicate that the larval gut is already colonized by bacteria at the onset of feeding (Makridis *et al.*, 2001). The present results, however, indicate that a well established gut community may not have developed in larvae until after 2-3 weeks of feeding. Elevated bacterial numbers and an unfavourable bacterial community of the live prey may therefore affect larvae to different extents, depending on the number of days spent in grazing on the live prey.

The results presented in ***Paper IV*** indicate a direct immunostimulatory effect of peptide-enrichment of the live prey, rather than indirect effects through modulation of the bacterial community. In agreement with previously published studies on larvae of various fish species (Balfry and Iwama, 2004; Grinde *et al.*, 1988; Lange *et al.*, 2001; Magnadottir *et al.*, 2005; Murray and Fletcher, 1976), low levels of C3 and lysozyme were already detected throughout the larvae at the onset of exogenous feeding. Stimulated production was furthermore observed as a result of offering peptide-enriched live prey to larvae. The results also suggested the maternal origin of IgM detected in larvae at the onset of feeding, not previously reported in halibut larvae. The maternal IgM was organized in clearly defined centres within the spleen, indicating an organized accrual of IgM molecules within this organ at early developmental stages. Offering

peptide-enriched live prey to larvae did not affect the measured concentration of IgM, with increasing levels measured in larvae after 29 days in feeding and onwards. This was relatively late compared to the reported IgM mRNA expression in halibut larvae after 16 days in feeding, but may coincide with the much later detection of IgM positive cells (Patel *et al.*, 2009). In agreement with the observations of Liang *et al.* (2006), the present results furthermore indicated that certain concentrations of the hydrolysate may have had immunostimulating effects, while higher concentrations may have had adverse effects. The results therefore emphasize the need for selecting the appropriate concentrations and treatment schedules during various developmental stages of the species.

Highly variable survival and overall success of first feeding larvae was observed in the present studies and has commonly been reported during intensive production of halibut larvae (Olsen *et al.*, 1999b; Ottesen and Olafsen, 2000; Shields *et al.*, 1999b; Verner-Jeffreys *et al.*, 2003a). *Vibrio wodanis* was identified in larvae from one incubator during the studies presented in ***Paper I*** and may have caused the elevated larval mortalities observed in this incubator. A product identified as *Vibrio* spp. (GeneBank accession number EU655423) was furthermore observed in larvae prior to increased larval mortalities observed in the respective incubators. Further attempts to relate the highly variable DGGE profiles or any single groups of bacteria identified to the overall larval success, however, revealed no relationship. Overall, the variable bacterial profiles of first feeding larvae observed in the present studies may be partly explained by the highly variable bacterial quality of the live prey. In agreement with previous findings (Verner-Jeffreys *et al.*, 2003b), only a part of the products observed in various samples of enriched *Artemia* was observed in first feeding larvae, supporting the hypothesis that a selection may occur, with only a part of the *Artemia*-associated bacteria able to establish themselves in the gut of larvae (Verner-Jeffreys *et al.*, 2003b). The present results may furthermore indicate that a certain period of offering live prey to larvae is needed to obtain community stability within the larval gut, as previously pointed out by Possemiers *et al.* (2004).

5.2.4 Live prey offered to larvae

The overall dominance of *Vibrio* spp. in the intestinal community of larvae after the onset of first feeding may be explained by the commonly observed dominance of *Vibrio* spp. in samples of the live prey analysed during the present studies (Korsnes *et al.*, 2006; Olsen *et al.*, 2000; Ritar *et al.*, 2004; Savas *et al.*, 2005). Products identical to the isolates used for bacterial treatment were found in samples of untreated as well as treated live prey, indicative of the live prey origin and a successful colonization of the respective groups as a part of the indigenous bacterial community of enriched *Artemia* nauplii (***Paper III***). Overall, the results indicate highly variable DGGE profiles in samples of the live prey, and also within the individual periods studied. In agreement with previously published studies (Olsen *et al.*, 2000; Verner-Jeffreys *et al.*, 2003b) *Pseudomonas*, *Pseudoalteromonas, Tenacibaculum, Acinetobacter, Moraxell* and *Corynebacterium* were among groups identified as a part of the dominating bacterial community in samples of the live prey. Previously published studies furthermore report the identification of *Alcaligens* and *Aeromonas* in samples of enriched *Artemia* (Olsen *et al.*, 2000), not observed in the present studies.

An analysis of unhatched and newly hatched *Artemia* cysts revealed the identification of *Vibrio*, with *Stenotrophomonas*, *Hydrogenophaga* and *Bacillus/Anoxybacillus* co-dominating the bacterial community. This may indicate a shift in the dominating bacterial community of the *Artemia* during enrichment, as previously suggested (Ritar *et al.*, 2004). In agreement with previous findings (Lee, 2003; López-Torres and Lizárraga-Partida, 2001), variable bacterial community structure was observed in *Artemia* from different sources. Analysis of the community structure during various developmental stages of the *Artemia*, furthermore revealed pattern profiles that indicate the presence of *Vibrio* in unhatched *Artemia* cysts. In contradistinction to previously published studies (López-Torres and Lizárraga-Partida, 2001; Verner-Jeffreys *et al.*, 2003b), the present studies therefore indicated that *Vibrio* species contaminating the live food rearing systems may be introduced with the *Artemia* cysts. The inability to isolate *Vibrio* from samples of *Artemia* cysts in the studies of Verner-Jeffreys *et al.* (2003b) may be partly explained by the use of *Artemia* from a different source or the presence of *Vibrio* in lower relative numbers due to unfavourable

environmental conditions that have been found to affect the activity and recovery of environmental vibrios (Mukamolova *et al.*, 2003).

6 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

In conclusion, a number of new findings are described in the present studies. Only a small part of the bacterial community in environmental samples has been found to be cultivable, but groups dominating the bacterial community were to a large extent found to be recoverable on traditional nutrient media. The identification of α-Proteobacteria in halibut larvae is described for the first time and a correlation was observed between the numbers of cultivable bacteria and the quality of unfed yolk sac larvae. Maternal IgM was furthermore detected in halibut larvae at the onset of feeding and was organised in defined centres within the spleen tissue. The results furthermore revealed reduced bacterial numbers during a critical stage in the production, when using inorganic clay as compared to marine microalgae for providing the environmental shading necessary during the first weeks of feeding. Improved environmental stability may be achieved through manipulation of the bacterial community and microbial manipulation of the live prey using autochthonous bacteria resulted in improved larval survival and may have positively affected larval growth. Successful stimulation of unspecific immune parameters and improved organ and tissue development of larvae were furthermore obtained through peptide-enrichment of the live prey.

The present studies have contributed new knowledge of the bacterial community of successful as well as unsuccessful halibut larvae produced in aquaculture. The results suggest a more heterogeneous bacterial community structure of unfed yolk sac larvae than previously reported and antagonistic activity was observed for bacteria dominating the cultivable community of successful larvae against groups dominating the cultivable bacterial community of larvae from units with poor overall success. The results, however, support the hypothesis that bacterial interaction(s) rather than the presence or absence of individual species or groups is of primary importance. The present results furthermore suggest that the highly variable quality of the live prey may affect the

development of the microbial community of larvae to a variable degree, depending on the developmental stage of larvae.

Future perspectives

The studies shed some light on the bacterial community structure during intensive production of halibut larvae and possible approaches for improving the overall production performances. Much work, however, remains to be done in order to understand more fully the dynamic bacterial community formed during various developmental stages and the interplay of various groups with respect to growth, survival and quality of commercially produced halibut larvae. For future recommendations, the selection of successful and reproducible methodology for analysis of the bacterial community is important. The DGGE method is a choice to be considered and the method may be successfully applied for analysis of the community diversity of bacteria and for analysing changes caused by various treatments. Some failures may always be expected, however, during the PCR amplification process and a general improvement and standardization of methodology, and the routine use of quality controls is advisable. Treatment of larvae through bioencapsulation of the live prey is recommended, as it represents minimal changes in the culture environment of larvae. Microbial manipulation during various developmental stages and the use of fish peptides for stimulation of innate immune parameters during a period representing the main bottleneck in the production of halibut larvae represent promising preventive methods that should be developed further.

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8 ORGINAL PAPERS