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# Selection and administration of probiotic bacteria to marine fish larvae

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Innovation and Creativity

# Acknowledgements

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## List of papers

### Paper 1

Fjellheim, A. J., Playfoot, K. J., Skjermo, J. and O. Vadstein. 2006. Large inter-individual variation in the dominant intestinal bacterial flora of reared cod larvae. Manuscript in prep. for Applied and Environmental Microbiology.

### Paper 2

Fjellheim, A. J., Playfoot, K. J., Skjermo, J. and O. Vadstein. 2006. The antagonistic bacterial flora of intensively reared cod larvae is dominated by *Vibrio*. Manuscript submitted to Aquaculture.

### Paper 3

Fjellheim, A. J., Klinkenberg, G., Skjermo, J., Aasen, I. M. and O. Vadstein. 2006. A comparison of candidate probiotic bacteria selected by two different screening strategies. Manuscript in prep. for Applied and Environmental Microbiology.

### Paper 4

Makridis, P., Fjellheim, A. J., Skjermo, J. and O. Vadstein. 2000. Control of the bacterial flora of *Brachionus plicatilis* and *Artemia franciscana* by incubation in bacterial suspensions. Aquaculture 185, 205-216.

### Paper 5

Makridis, P., Fjellheim, A. J., Skjermo, J. and O. Vadstein. 2000. Colonization of the gut in first feeding turbot larvae (*Scophthalmus maximus* L.) by bacterial strains added to the water or bioencapsulated in rotifers (*Brachionus plicatilis*). Aquaculture International, 8, 367-380.

### Paper 6

Fjellheim, A. J., Skjermo, J. Østensen, M. A. and O. Vadstein. 2006. An *in vivo* test of probiotic candidates from the dominant and the antagonistic bacterial flora of cod larvae (*Gadhus morhua* L.). Evaluation of colonization efficiency and vitality of larvae. Manuscript in prep. for Aquaculture.

## Introduction

The global aquaculture production has been steadily increasing over the last years. In 2004 the total production was over 28 million tonnes and valued nearly 37 billion US$. The production of marine fish in Europe was 130 thousand tonnes and from this the share of turbot was more than six thousand tonnes and the share of Atlantic cod was almost four thousand tonnes (FAO). Intensive aquaculture production of marine fish larvae has been hampered due to low and unpredictable survival during first-feeding. The larvae are reared at high densities and are exposed to substantial bacterial levels during first feeding (Olafsen, 2001). The intensive rearing conditions provide an environment selecting for opportunistic bacteria (Skjermo et al. , 1997) that may lead to reduced growth and survival of the larvae (Salvesen et al. , 1999). Supply of organic material during hatching results in growth of opportunistic bacteria in the tanks (Vadstein et al. , 2004). In addition, opportunistic bacteria are associated to the live feed (Paper 4). Microbial management in marine larviculture involves several measures (Skjermo and Vadstein, 1999) (Figure 1). The rearing water can be modified by microbial maturation, which reduces the number of opportunistic bacteria (Skjermo et al. , 1997), or by water recirculating systems that stabilize the microbial community and create a more robust environment that is less susceptible to disturbances (Attramadal, 2004). The use of immunostimulants can boost the non-specific immune system (Vadstein, 1997; Conceicao et al. , 2001) of fish larvae, and enhance the resistance against pathogens (Skjermo and Bergh, 2004). In addition, the vitality of fish larvae can be improved by the addition of probiotics to the water or the feed, with the objective of colonizing larvae with beneficial bacteria (Gatesoupe, 1999; Irianto and Austin, 2002; Vine et al. , 2006).
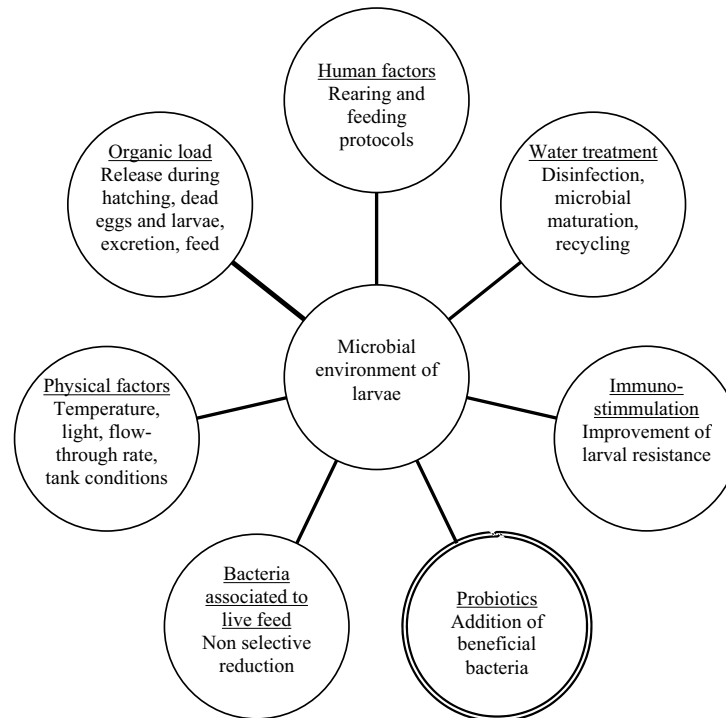
**Figure 1.** Factors that can influence the microbial environment experienced by marine larvae during first feeding.

The increasing interest in probiotics for use in aquaculture production has lead to investigations of probiotic preparations for live feed, crustaceans, molluscs and fish production (Verschuere et al. , 2000). Several definitions of probiotics have been made (Gram and Ringø, 2005), and the definition proposed by Fuller (1989) is "live microbial feed supplements which beneficially affect the host animal by improving its intestinal microbial balance". To fully understand the effects of probiotic organisms it is important to know by which mechanisms they benefit the host. Several mechanisms have been put forward as important for probiotic bacteria;

►Production of inhibitory compounds
►Competition for adhesion sites

► Enhancement of immune response

► Production of beneficial compounds, such as digestive enzymes and vitamins

► Antiviral effects

► Improvement of water quality

These mechanisms are not mutually exclusive. The probiotic organisms should also be able to attach and grow in intestinal mucus, and tolerate the gut environment, to be able to colonize the host. Probiotic candidates must be tested in experimental exposure tests, to assure that they are not detrimental to the host. *In vivo* information on the host-microbe interactions, are important to get a better understanding of these mechanisms.

In the search for probiotic bacteria, different selection strategies have been applied in studies on marine fish larvae (Gram and Ringø, 2005). Probiotic candidates are mostly chosen because they show *in vitro* inhibitory activity against a target pathogen. Also, the dominant intestinal bacterial flora in larvae from well performing rearing groups has been regarded as probiotic candidates (Huys et al. , 2001). The dominant bacterial flora is regarded capable of excluding invading bacteria from the adhesion sites of the gut wall (Verschuere et al. , 2000). In addition, bacterial adhesiveness to mucus and growth properties in mucus has been used as an initial selection strategy of probiotics (Vine et al. , 2004a-b).

The aim of this thesis was to make a rational selection of probiotic bacteria and to test the candidates *in vivo* by controlled colonization in first feeding experiments with marine larvae. In order to achieve this, the following issues were investigated:

► The dominant and the antagonistic intestinal bacterial flora of cod larvae from different rearing conditions were characterized, to obtain a pool of isolates.

► Bacteria from the pool of isolates were evaluated using several *in vitro* tests, to screen the isolates regarding their potential as probiotics.

►Cod larvae were exposed to the probiotic candidates in a small-scale *in vivo* experiment to evaluate if the bacteria were detrimental or beneficial to larvae.

►The bioencapsulation of bacteria in live feed was studied to enable effective transfer of probiotic bacteria to fish larvae.

►Two first-feeding experiments with different marine fish species were conducted to see if controlled colonization could be obtained, and to see if the selected probiotic candidates had beneficial effects *in vivo*.

## Methods to characterize microbial communities and to detect and quantify added bacteria

Different methods are used to characterize microbial communities and to detect the presence of specific bacteria. Some methods rely on culture techniques and phenotypic characterization (Hansen and Sørheim, 1991; Bagge-Ravn et al. , 2003; Paper 1; Paper 2), others on immunological techniques (Kotani and Mcgarrity, 1986; Adams, 1991; Paper 4; Paper 5), and lately DNA based methods are expanding in this area of research (Zoetendal et al. , 2004; Dumonceaux et al. , 2006; Paper 1; Paper 6). The two immunological methods immunocolony-blot (ICB) and enzyme-linked immunosorbent assay (ELISA), and the DNA based method terminal restriction fragment length polymorphism (T-RFLP) were applied in this thesis. These methods were used to detect the presence of specific bacteria. In addition, culture techniques followed by phenotypic characteriazation and sequence analysis of a part of the 16S rDNA, as well as T-RFLP were used to characterize bacterial communities in association to marine larviculture (water, rotifers and larvae).

A large number of tests can be used to characterize bacteria phenotypically. In this thesis, Gram reaction, oxidase reaction, fermentative ability, motility, shape and several other criteria were used to group the bacteria into clusters. The results from phenotypic tests can also be used to place the bacteria directly into genera by using identification shemes (Bagge-Ravn et al. , 2003).

Antibody specific for the added bacteria is used by the immunological methods to detect surface structures of specific bacteria. The production of this specific antibody can only be done with cultivable bacteria. The bound antibody is made visual through colour reactions. The main difference between ICB and ELISA, is that ICB is more laborious, however, ELISA has a higher detection level compared to ICB (Paper 4; Paper 5).

T-RFLP was developed to analyse microbial community structure and dynamics (Liu et al. , 1997; Osborn et al. , 2000), however it has also been used to track specific bacteria in different ecosystems (Jernberg et al. , 2005; Jung et al. , 2005). To run T-RFLP, DNA from a sample is the template for the PCR, and one or both of the primers are labelled with a fluorescent dye. The amplified products are digested with one or more restriction enzymes, and the size of the fluorescent terminal fragment is determined. In this thesis, the method was used both to analyze microbial communities (Paper 1; Paper 6) and to detect specific bacteria that were added to the rearing system of cod larvae (Paper 6) (Figure 2). About 500 bp of the 16S rDNA was amplified with real-time PCR prior to the T-RFLP analysis. Real time PCR was used to enable detection of the late logarithmic amplification phase. By stopping the DNA amplification in this phase, the risk for changing the ratios between different bacterial templates was lowered (Suzuki and Giovannoni, 1996; Kanagawa, 2003). To enable detection of specific bacteria, the expected and observed terminal restriction fragments (TRF) of the added bacteria was determined. The TRF of the added bacteria was defined as the observed TRF±1 bp. This variability was included because variation between observed TRF lengths has been found in replicate runs of the same sample (Kaplan and Kitts, 2003). The percentage of this specific TRF related to the total peak area was calculated by the variable threshold method (Osborne et al. , 2006). As the case was for the immunological methods, also T-RFLP was hampered by "background noise" created by other bacteria in the experimental system.
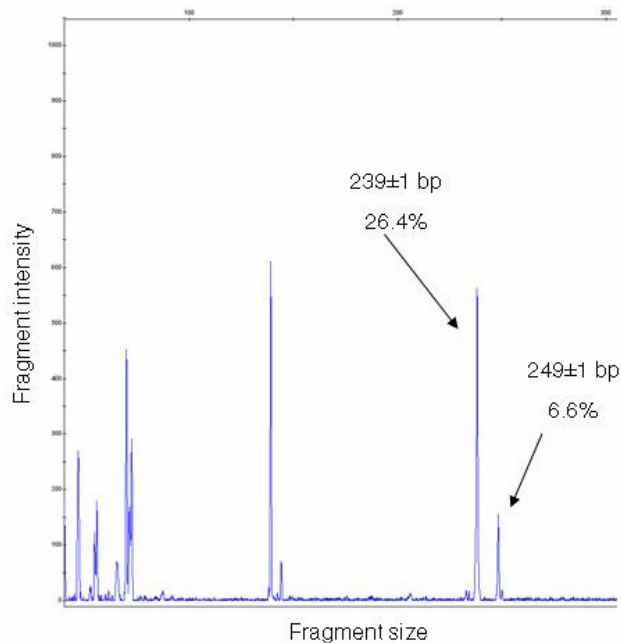
**Figure 2.** Example of a T-RFLP profile from rotifers in a tank that was added the antagonistic probiotic candidates *Pseudoalteromonas* (RA7-14; 239±1 bp) and *Roseobacter* (RA4-1; 249±1 bp). Each peak represents one or several species of bacteria.

## The dominant and the antagonistic intestinal bacterial flora of marine fish larvae

The intestinal bacterial flora of several species of marine fish larvae has been characterized (Muroga et al. , 1987; Hansen et al. , 1992; Blanch et al. , 1997; Nedoluha and Westhoff, 1997; Eddy and Jones, 2002; Verner-Jeffreys et al. , 2003), and a shift in the bacterial flora, regarding composition and quantity, is found with larval age, nutritive status and environmental conditions (Hansen and Olafsen, 1999; Ringø and Birkbeck, 1999). Marine larvae drink water one day after hatching (Mangor Jensen and Adoff, 1987; Reitan et al. , 1998), thus the intestinal bacterial flora is influenced by bacteria in the surrounding water at an early stage. The number of intestinal bacteria

increases as the larvae start eating (Huys et al. , 2001; Eddy and Jones, 2002) and the composition is influenced by the bacterial flora associated to live feed (Muroga et al. , 1987; Eddy and Jones, 2002). The functions of the intestinal bacterial flora are nutrient processing (Bairagi et al. , 2002), contribution to digestive processes (Rowland, 1992), maturation of the innate immune system (Hansen and Olafsen, 1999) and prevention of pathogen colonization (Verschuere et al. , 2000). In addition, many genes are up- or down-regulated in response to the intestinal bacterial flora (Rawls et al. , 2004) with implications that are mostly unknown. Thus, the intestinal bacterial flora is of great importance and probably influences development, growth and survival in marine larvae. Most studies on the intestinal bacterial flora of fish larvae have used traditional methods and culture techniques. However, in the last decade several molecular methods have been applied (Zoetendal et al. , 2004). These methods can be used to characterize the total bacterial flora as well as the cultivable bacterial flora, and will undoubtedly be important to fully understand the ecology of the intestinal bacterial flora.

In order to describe and compare the bacterial flora between individuals and rearing groups, the dominant cultivable intestinal bacterial flora of Atlantic cod larvae from different rearing groups (Paper 1) was phenotypically characterized (Figure 3). For some of these isolates the 16S rDNA was partially sequenced. In addition, the culture independent approach T-RFLP was used to characterize the total bacterial flora. The bacterial flora varied qualitatively and quantitatively between individuals from the same rearing condition and between cod larvae from different rearing conditions. Inter-individual variation is also found in human beings (Zoetendal et al. , 1998; Hopkins et al. , 2002), pigs (Simpson et al. , 2000) and broiler chickens (Zhu et al. , 2002), and may be due to stochastic factors occurring during bacterial colonization (Verschuere et al. , 1997). The inter-individual variation could also be caused by genetic differences between individuals (Vaahtovuo et al. , 2003). The cultivable bacterial flora in larvae from two hatcheries was dominated by bacteria with 16S rDNA similar to the Gram positive *Microbacterium. Microbacterium* was also found to a less extent in Atlantic cod larvae by Korsnes et al. (2006), but is rarely found in the bacterial flora of marine fish larvae. All other dominant isolates had 16S rDNA similar to Gram negative bacteria. The γ-Proteobacteria was most common, and represented by *Marinomonas*, *Vibrio*,

*Pseudomonas*, *Pseudoalteromonas*, *Neptunomonas* and *Halomonas*. In addition, *Roseobacter* within the α-Proteobacteria and two isolates probably within the Bacteroides and Cytophaga group were detected. The T-RFLP analysis revealed considerably higher diversity than the culture based phenotypic characterization. However, the detection level of the culture technique was not below 3%, thus if only TRFs that represented more than 3% of the total peak area were used in the comparison, similar diversity was found.
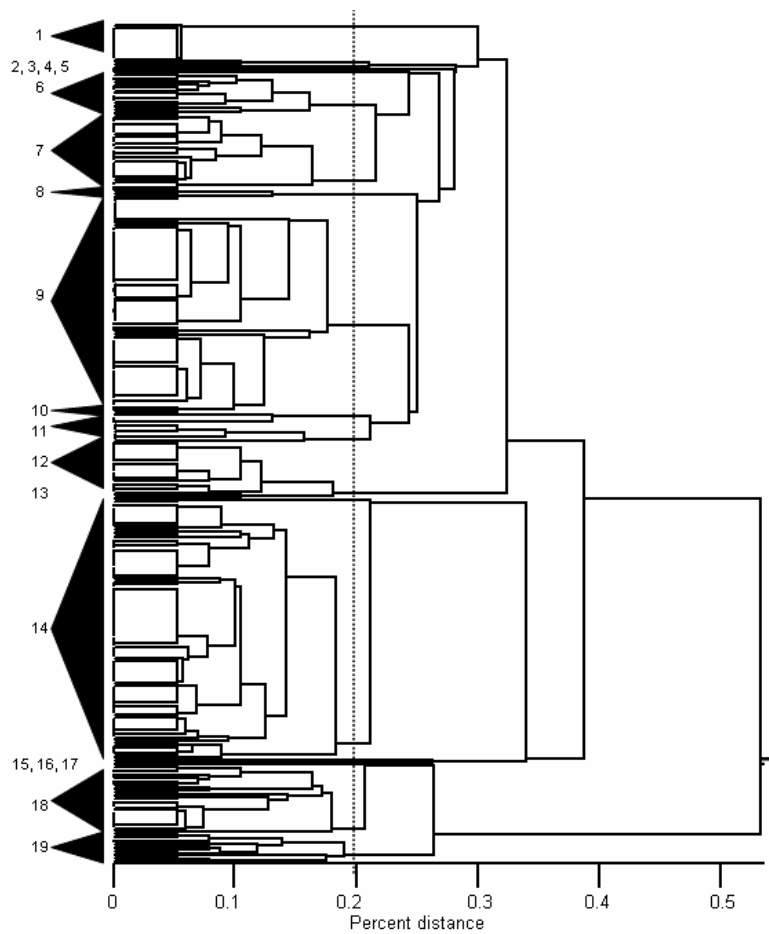


**Figure 3.** Cluster diagram based on phenotypic characterization for all dominant isolates using average linkage and percent distance. The isolates were clustered at 80% similarity as indicated by the dotted line. Numbers on the left indicate the different clusters obtained.

Bacterial antagonism is common among bacteria from the marine environment (Long and Azam, 2001; Grossart et al. , 2004) and among bacteria from marine fish larvae (Westerdahl et al. , 1991; Sugita et al. , 2002; Makridis et al. , 2005a). Bacteria from different genera (Sugita et al. , 1996; Ringo and Gatesoupe, 1998; Long and Azam, 2001; Hjelm et al. , 2004b; Makridis et al. , 2005a) (Figure 4) produce chemical substances that cause antagonistic activity, including antibiotics, organic acids, hydrogen peroxide, siderophores and bacteriocins. A few percent of the cultivable bacterial flora from fish is usually antagonistic to pathogenic bacteria *in vitro* (Sugita et al. , 1996, 2002; Hjelm et al. , 2004a), although in some cases, higher percentages of antagonistic bacteria have been reported (Westerdahl et al. , 1991; Makridis et al. , 2005a). However, the importance of antagonistic interactions *in vivo*, are not well documented (Atlas, 1999). Under *in vitro* conditions bacterial antagonism is medium-, growth phase- and temperature- dependent (Bizani and Brandelli, 2004; Hjelm et al. , 2004a; Monteiro et al. , 2005), and can even depend on static growth conditions (Bruhn et al. , 2006). Thus, it can be questioned whether conditions suitable for production of inhibitory compounds will occur in the intestinal tract of fish. On the other hand, the fact that so many marine bacteria have this property, indicates that it involves competitive advantages, at least under some circumstances.
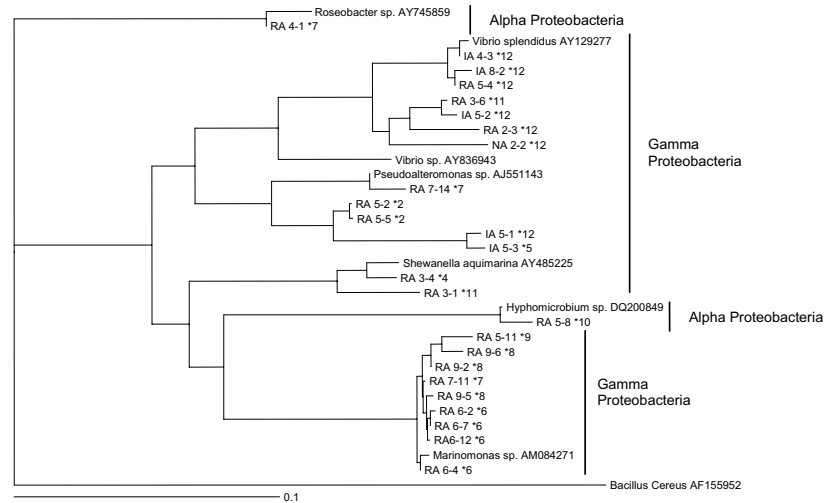
**Figure 4.** Phylogenetic tree based on the bacterial 16S rDNA sequences recovered from antagonistic bacteria. The scale bar indicates 10% sequence difference. The 16S rDNA sequence of *Bacillus cereus* (AF155952) was used as outgroup reference, and accession numbers of reference sequences are indicated. The number after the asterisk refers to the cluster where isolates were placed according to the phenotypic characterization (Paper 2).

In this thesis, the cultivable bacterial flora in cod larvae from five rearing groups was screened for inhibitory activity against *Listonella anguillarum* (strain HI610) by using the replica plating method (Paper 2). Most of the antagonistic bacteria were members of the γ-Proteobacteria, represented mainly by isolates with 16S rDNA similarity to *Vibrio* and *Marinomonas*, but also isolates with sequence similarity to *Pseudoalteromonas* and *Shewanella* were found (Figure 4). *Vibrio* constituted a major part of the antagonistic intestinal bacteria in Japanese flounder (Sugita et al. , 2002), and was the only antagonistic bacterial genus in the intestines of Senegalese sole (Makridis et al. , 2005a). *Pseudoalteromonas* has shown a broad range of inhibitory activities against both bacteria and other organisms (Holmström et al. , 2002). Members of α-Proteobacteria also inhibited the growth of *L. anguillarum,* and were represented by *Hyphomicrobium* and *Roseobacter*. The most numerous antagonistic bacteria in a turbot rearing system were *Roseobacter* spp. (Hjelm et al. , 2004b). In addition, the antagonistic *Roseobacter*

sp. strain 27-4 has demonstrated probiotic potential *in vivo* (Hjelm et al. , 2004a; Planas et al. , 2006). Both the species composition and the percentage of antagonistic isolates in the intestinal bacterial flora (Figure 5) varied considerably between individual cod larvae from the same rearing condition and also between rearing conditions. Inter-individual variation regarding the share of intestinal bacteria with inhibitory activity was also found in turbot (Westerdahl et al. , 1991).
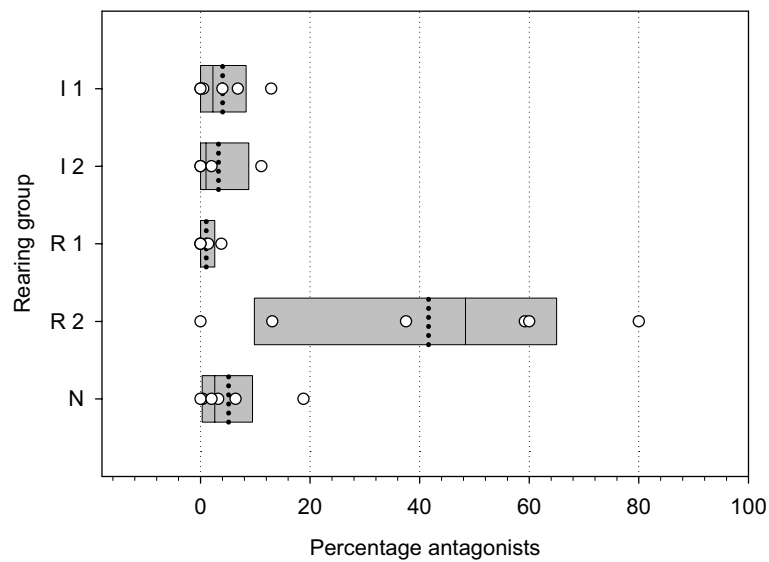


**Figure 5.** Percentage antagonistic isolates of total colony forming units in individual larva from five different rearing groups. Cicles (○) indicate individual larvae, the boxes show 25th and 75th percentiles and median. The dotted lines are the mean values.

The number of cultivable bacteria varied up to 440 times between larvae from the same rearing tanks, and variation was also found between larvae from different rearing conditions (Figure 6). Quantitative differences regarding cultivable bacteria have also been found in rainbow trout (Spanggaard et al. , 2000) and Japanese coastal fish (Sugita et al. , 2005). The total number of bacteria associated to the fish larvae was also compared between samples. The bacterial DNA from the cod larvae was amplified with

real time PCR, and a threshold cycle (Ct) was determined for each sample. The Ct value allows for accurate and reproducible quantification of the bacterial DNA. The Ct values varied from 22.9 to 27.6 cycles, and with 80% DNA amplification efficiency this corresponds to up to 16 times difference between individual larvae. Thus, in quantitative terms, both the cultivable and the total number of bacteria varied between individual larvae. However, the cultivable bacteria varied far more than the total bacterial numbers. In addition, it was found that increasing total numbers of bacteria corresponded to decreasing CFU (Figure 7). This can be explained if fish larvae can harbour only a certain amount of bacteria. Thus, increasing numbers of uncultivable bacteria will displace the cultivable bacteria.
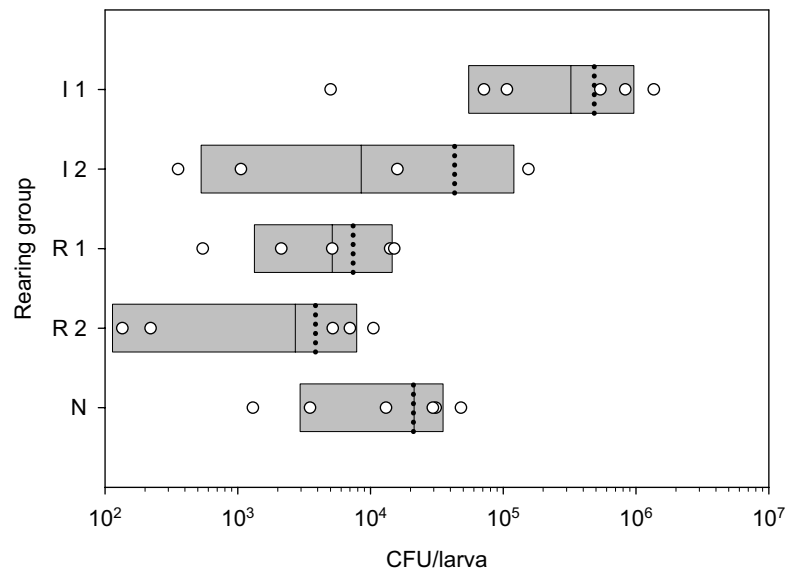


**Figure 6.** CFU in individual cod larvae from five different rearing groups.
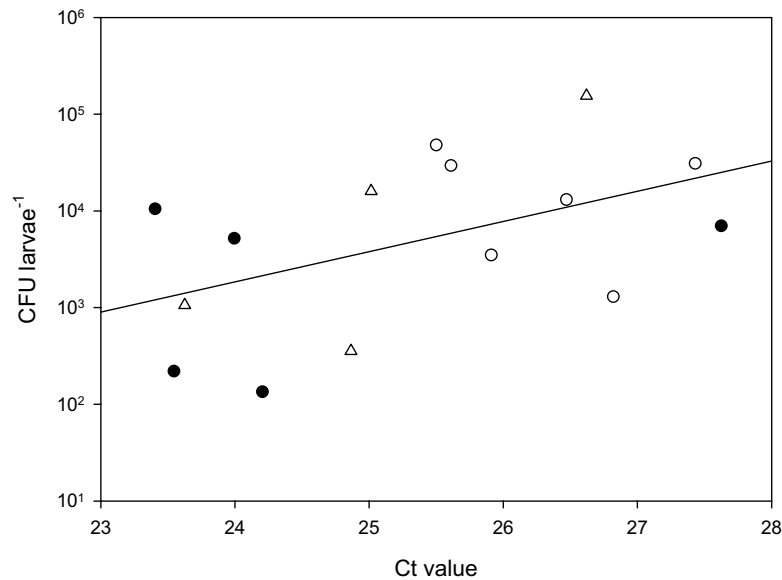
**Figure 7.** Ct (Threshold cycle) value found in real time PCR vs colony forming units (CFU) in individual larvae. The 95% confidence interval for the slope of the fitted line is    -0.018 to 0.643. ($\triangle$) larvae from hatchery I, ($\bigcirc$) larvae from hatchery N, and ($\bullet$) larvae from hatchery R.

## *In vitro* probiotic tests

A good pool of probiotic candidates is vital when searching for probiotic bacteria (Verschuere et al. , 2000). Nearly 500 bacterial isolates from the intestinal bacterial flora of cod larvae from different rearing conditions were isolated as a pool of candidate bacteria in this thesis. The isolates were obtained by two selection strategies, i.e. the dominant and the antagonistic bacterial flora of healthy cod larvae. After the phenotypic characterization, about 10% of the isolates were chosen for evaluation of probiotic properties. These isolates were chosen based on uniqueness, fermentative ability and dominance. An attempt was made to represent the diversity recorded in the phenotypic characterization, but with the goal of reducing the total number of isolates. In addition we wanted to end up with comparable numbers of dominant and antagonistic isolates.

In order to further reduce the number of probiotic candidates it is important to screen the isolates for beneficial properties that are important to probiotic bacteria. Screening for bacterial antagonism against a target pathogen is the most common method used (Gram and Ringø, 2005). However, screening for antagonism against dominant bacterial strains, to see if the normal bacterial flora is inhibited, is also relevant. The dominant bacteria were found to be less inhibited in the well diffusion agar assay (WDAA), compared to the pathogenic bacteria. The abilities of bacteria to attach and grow in mucus are also screened for (Olsson et al. , 1992; Vine et al. , 2004a-b; Chabrillon et al. , 2005). These are important properties to exclude pathogenic bacteria in the gut, and to enable colonization and persistence in the intestinal tract. In addition, probiotic bacteria may help its host by contributing to digestive processes in the gut (Rowland, 1992), as the intestinal bacterial flora in fish produces extracellular enzymes (Bairagi et al. , 2002; Ramirez and Dixon, 2003). Probiotic bacteria should also be resistant to bile to persist in the digestive tract (Nikoskelainen et al. , 2001), and they should tolerate low pH to pass through the acidic environment in the stomach. However, resistance to acid is not required for probiotic candidates aimed at marine larvae, as the digestive system is alkaline during first feeding (Hoehne-Reitan et al. , 2001). In addition to beneficial properties, also detrimental properties should be screened for. Haemolytic bacteria are found in large numbers in the rearing of marine larvae (Westerdahl et al. , 1991; Salvesen et al. , 1999; Olsen et al. , 2000). Haemolysis is often connected to pathogenic bacteria (Austin et al. , 2005), thus it seems wise to avoid the use haemolytic strains as probiotics.

In this thesis, the WDAA was used to evaluate antagonism against four pathogenic bacteria (Paper 3). In addition, antagonism against three dominant isolates from cod larvae was tested. A large proportion (52%) of the isolates that inhibited the growth of *L. anguillarum* (strain HI610) in the initial screening (replica plating method), did not inhibit the growth of this strain in the WDAA. It was not checked if this was due to the loss of antagonistic activity during storing or because of differences in the two tests. A pathogenic strain that is used in the initial search for antagonistic bacteria, should preferably be inhibited by a wide array of strains. In the WDAA, the pathogenic *Vibrio logei* was inhibited by 23 probiotic candidates, whereas *L. anguillarum* (strain HI610)

was inhibited by 16 candidates. Thus, *Vibrio logei* might have been more effective in finding antagonistic bacteria in the initial screening. The pathogenic *Marinomonas* sp., on the other hand, was only inhibited by five probiotic candidates. Two of the three dominant isolates we tested antagonism against had DNA similarity to *Microbacterium*, while the third had DNA similarity to *Marinomonas*. The two dominant *Microbacterium* strains were inhibited by strains with DNA similarity to *Marinomonas* (and one *Vibrio*), whereas the *Marinomonas* was inhibited by stains with DNA similarity to *Roseobacter* and *Vibrio*. Thus, the inhibition pattern against the dominant strains was strain-specific to some degree.

The ability of bacteria to attach to intestinal mucus is regarded important for the colonization of the intestine (Balcazar et al. , 2006; Vine et al. , 2006), and has been used as a selection criterion for probiotic bacteria in previous studies (Olsson et al. , 1992; Vine et al. , 2004b; Chabrillon et al. , 2005). In the present study (Paper 3), the adhesion to intestinal mucus of cod was similar between the bacterial isolates from the dominant and from the antagonistic selection strategies (Figure 8). Less than 5% adhesion to cod intestinal mucus was found with the majority of the isolates, however, isolates with equal to or higher than 5% binding to mucus were a diverse group with 16S rDNA similarities to *Marinomonas*, *Microbacterium*, *Pseudoalteromonas*, *Pseudomonas*, *Roseobacter* and *Vibrio*. Highest ability to attach to mucus, considering both the dominant and antagonistic bacterial groups, was found among isolates with 16S rDNA similarity to *Vibrio*. *Vibrio* has also been found to have good adhesive abilities to sole mucus (Chabrillon et al. , 2005).
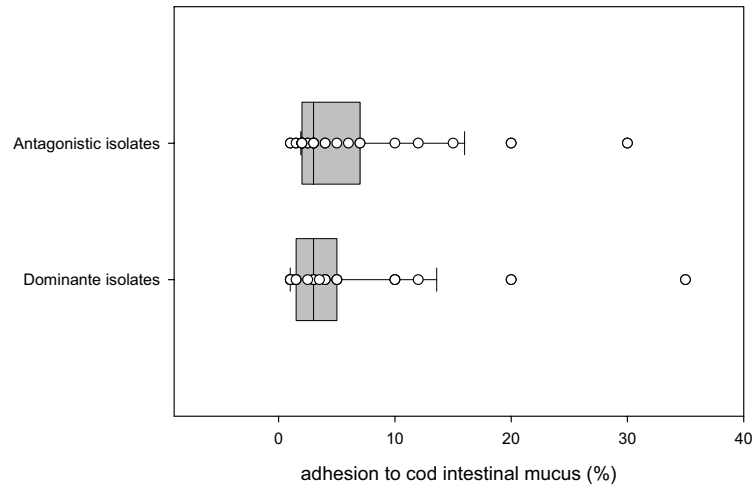
**Figure 8.** Adhesion to cod mucus for bacterial isolates selected by the dominant or the antagonistic selection strategy.

Bacterial growth characteristics have also been used to screen probiotic bacteria, and it has been proposed that an *r*-selected probiotic bacterium with a short lag phase and a fast growth rate in intestinal mucus will be the most competitive *in vivo* (Vine et al. , 2004a). Although probiotic bacteria with these growth characteristics might have better competitive abilities, it was found that turbot larvae grew better when the proportion of fast-growing bacteria in the rearing water was low (Salvesen et al. , 1999). In this thesis, the majority of isolates with higher maximum specific growth rates than 0.075 $h^{-1}$ had sequence similarity to *Vibrio* (eight isolates), but also three isolates with sequence similarity to *Pseudoalteromonas* grew faster than 0.075 $h^{-1}$ (Figure 9). On the other hand, the isolates with 16S sequence similarity to *Roseobacter*, did not grow at all in cod intestinal mucus.
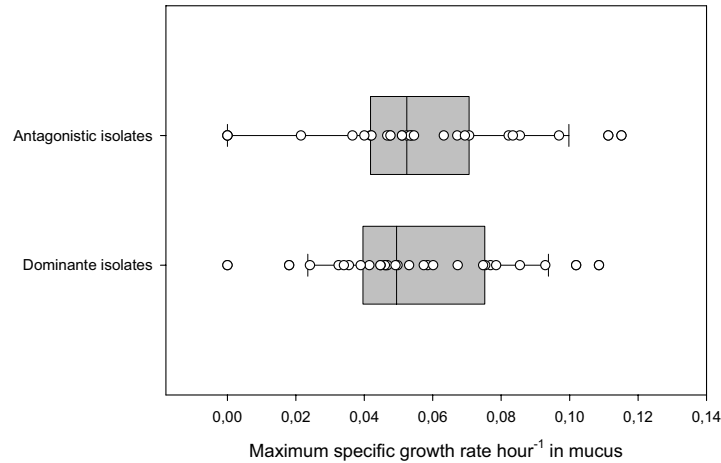
**Figure 9.** Growth rate in cod mucus for bacterial isolates selected by the dominant or the antagonistic selection strategy.

Extracellular enzymes are produced by the intestinal bacterial flora in fish (Bairagi et al. , 2002; Ramirez and Dixon, 2003; Paper 3). In bacteria from the intestinal tract of cod larvae, the production of constitutive extracellular enzymes varied between bacteria from the dominant and the antagonistic selection strategies, but was not significantly different between the two groups (Paper 3). Most isolates had high phosphatase and leucine arylamidase activity, whereas varying activity was recorded for esterase (C4), esterase lipase (C8), valine arylamidase, phosphohydrolase, β-galactosidase, α-glucosidase, β-glucosidase and glucosaminidase. Atlantic cod larvae probably have limited ability to digest carbohydrates (Perez-Casanova et al. , 2006), however, bacteria from the intestinal bacterial flora of Atlantic cod larvae produce several extracellular enzymes that degrade carbohydrates (Paper 3). In juvenile turbot the bacterial flora is involved in protein degradation in the distal segments of the gut (De Schrijver and Ollevier, 2000) and production of short chain fatty acids by bacteria can contribute to the metabolism of the host (Rowland, 1992). The human microbiome harbours significantly enriched metabolism of glycans, amino acids, and xenobiotics (Gill et al. , 2006), and this could be the case for marine fish as well.

Probiotic bacteria must be able to survive the transit to the gastrointestinal tract (Balcazar et al. , 2006). In the present study, all the bacterial isolates tolerated exposure to cod bile (Paper 3). The physiological concentration of bile in the fish intestine was not known, however, the 10% bile concentration that was used in the test is probably relatively high compared to what is found in the intestinal tract. The fish bile test indicated that all the bacterial isolates may persist in the gut with the presence of bile.

High proportions of bacteria from both selection strategies were found to be haemolytic in the present study. As the case for pathogenic vibrios (Austin et al. , 2005), only β-haemolytic isolates were found. The haemolytic isolates had 16S rDNA similar to *Marinomonas*, *Microbacterium*, *Pseudoalteromonas*, *Pseudomonas*, *Shewanella* and *Vibrio*, thus a wide range of bacteria have this property. Haemolytic bacteria are often found in large quantities in the rearing of marine larvae (Westerdahl et al. , 1991; Salvesen et al. , 1999; Olsen et al. , 2000), however, countermeasures may lead to a significant reduction in their numbers (Olsen et al. , 2000). Expression of haemolytic activity is dependent on bacterial growth phase, temperature and pH (Munn, 1978), and it can be differently expressed on blood from different animal species (Austin et al. , 2005). These factors were not investigated for the isolates in the present study. Haemolysin can be a virulence factor, and therefore all isolates with this activity were excluded as probiotic candidates. However, one of the seven isolates that were chosen for further testing was a case of doubt as a very weak clearing zone on blood agar was detected after 10 days of incubation. In the experimental exposure test this isolate (ID4-29) was detrimental to the fish larvae.

Based on the *in vitro* tests presented above, seven bacterial isolates (Table 1) were chosen for further evaluation of probiotic properties in *in vivo* experiments with cod larvae. The chosen strains performed well in one or several of the *in vitro* tests. Fermentative ability was found in three of the isolates. Antagonism was only found in isolates from the antagonistic selection strategy, and isolates with antagonism against several pathogens were preferred. Isolates with high adhesion to mucus were preferred, but also isolates with low adhesion were included if they performed well in other tests. The production of extracellular enzymes and the growth rate in mucus varied between

the chosen isolates. We regarded the production of several extracellular enzymes as a positive characteristic, whereas the growth rate in mucus was less emphasized in the selection. Bacteria with high growth rates have competitive advantages in habitats with surplus of space and food, however, opportunistic bacteria can be detrimental to marine larvae (Skjermo et al. , 1997; Salvesen et al. , 1999).

**Table 1.** Properties of the bacterial isolates that were selected as probiotic candidates based on evaluation of *in vitro* test results. Antagonism was tested against three dominant isolates and four pathogens, the number of isolates that was inhibited is listed. The total score is shown for extracellular enzyme production, maximum score is 95, 5 for each of 19 enzymes . * very weak clearing zone after 10 days of incubation on blood agar.

| | RA 7-14 | RA 4-1 | RA 3-6 | ND2-7 | ID 3-10 | RD 5-30 | ID 4-29 |
|---|---|---|---|---|---|---|---|
| Candidate bacterium ID with closest match in Genbank | *Pseudoaltero-monas* sp. | *Roseobacter* sp. | *Vibrio ordalii* | *Micro-bacterium* sp. | *Micro-bacterium* sp. | *Vibrio gallicus* | *Vibrio ordalii* |
| Selection strategy | Antagonistic bacterial flora | Antagonistic bacterial flora | Antagonistic bacterial flora | Dominant bacterial flora | Dominant bacterial flora | Dominant bacterial flora | Dominant bacterial flora |
| Antagonism to dominant isolates | 1 | 1 | 2 | 0 | 0 | 0 | 0 |
| Antagonism to pathogens | 3 | 4 | 4 | 0 | 0 | 0 | 0 |
| Adhesion to mucus (%) | 2 | 20 | 10 | 1.5 | 5 | 4 | 35 |
| Growth rate in mucus | 0.097 | No growth | 0.083 | 0.033 | 0.039 | 0.075 | 0.034 |
| Extracellular enzyme production | 47 | 22 | 35 | 42 | 47 | 22 | 32 |
| Tolerance to cod bile | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| Haemolytic activity | No | No | No | No | No | No | No* |
| Fermentative ability | No | No | Yes | No | No | Yes | Yes |

## Small scale *in vivo* evaluation of probiotic candidates

A probiotic candidate bacterium that is promising based on *in vitro* properties, needs to be tested *in vivo* to confirm positive (or negative) effects on fish larvae (Bergh et al. , 1992; Makridis et al. , 2005b). The experimental exposure test is laborious compared to the *in vitro* tests, and therefore only a limited number of bacterial isolates can be chosen for testing. However, compared to a first feeding experiment, the experimental exposure test is far less laborious. Thus, the experimental exposure test is a valuable tool to evaluate probiotic bacteria *in vivo*, and specifically to avoid the use of pathogenic bacteria in first-feeding experiments.

In the experimental exposure test, cod eggs were surface disinfected and distributed one by one in small wells with autoclaved seawater (Paper 3). The probiotic candidates were added to the water at low ($10^4$ bacteria ml$^{-1}$) and high density ($10^7$ bacteria ml$^{-1}$). Surface disinfected cod eggs in non-autoclaved seawater served as a positive control, whereas the negative control included addition of the pathogenic *L. anguillarum* (strain HI610) into the water. The fish larvae were not fed during the experiment, therefore all larvae eventually died due to starvation. The mortality of larvae was different between treatments (Figure 10). *L. anguillarium* (HI 610) and two of the probiotic candidates, were detrimental to the cod larvae. Both probiotic candidates that were harmful to the larvae had 16S rDNA similarity to *Vibrio ordalii* and to the pathogenic *L. anguillarum*. These two probiotic candidates had *in vitro* properties that are considered important for probiotic bacteria, but were both rejected as a result of the experimental exposure test. Five probiotic candidates performed significantly better than the positive control in the experimental exposure test, both when added at low and high density. Thus, these five bacteria were chosen for use in a first feeding experiment. The results showed that the bacterial species present, influenced mortality to a large extent, whereas the density of bacteria was less relevant. Similar mortality rates, independent of bacterial density, was also found for sea bream larvae (Makridis et al. , 2005b). In conclusion, the experimental exposure test is a simple test that gives valuable information regarding the impact of the added bacteria on fish larvae. In addition, the results indicate that the test might be further simplified by testing only one bacterial density.
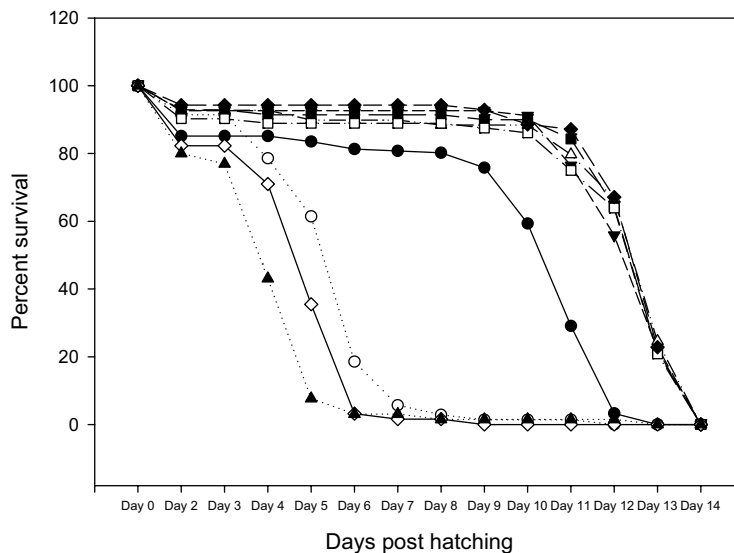
**Figure 10.** Survival of cod larvae when they were *in vivo* exposed to seven probiotic candidates, a negative and a positive control at high bacterial density ($10^7$ ml$^{-1}$). Negative control was *L. anguillarum* HI 610 (▲), positive control was non-sterilized rearing water (●) ($5.9 \times 10^2$ bact ml$^{-1}$). Probiotic candidates: RA 3-6 (O, *Vibrio*), RA 4-1 (▼, *Roseobacter*), RA 7-14 (△, *Pseudoalteromonas*), ID 3-10 (■, *Microbacterium*), RD 5-30 (□, *Vibrio*), ND 2-7 (◆, *Microbacterium*), ID 4-29 (◇, *Vibrio*).

## Methods to transfer probiotic bacteria to fish larvae

Effective transfer of probiotic bacteria to fish larvae is vital for the colonization potential of the bacteria. The addition of bacteria to the rearing water at an early stage is one method that has proven effective (Ringø et al. , 1996; Ringø and Vadstein, 1998; Paper 5). Another way of transferring bacteria to fish larvae is by bioencapsulating bacteria in the live feed (Gatesoupe, 1994; Munro et al. , 1999; Paper 5). This technique also ensures exchange of the possibly detrimental flora of the live feed organisms before delivery to fish larvae. *Brachionus plicatilis* and *Artemia franciscana* are both filter feeders (Vadstein et al. , 1993; Makridis and Vadstein, 1999) that are commonly used live feed in the intensive rearing of marine larvae.

In this thesis, it is shown that rotifers grazing in bacterial suspensions were able to bioencapsulate added bacteria within 20 minutes (Figure 11). The bioencasulation was more

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efficient at high bacterial concentrations, and depended on whether bacteria were added alone or in mixture (Paper 4). Bioencapsulation efficiency by *A. franciscana* varied between the two bacterial strains that were tested. One of the bacterial strains was effectively bioencapsulated within 30 min, whereas the other strain was bioencapulated within 60 min. To promote colonisation of the fish larvae with the bioencapsulated bacteria, the bacteria should persist in the live feed for some time after transfer to fish tanks. The transfer of *B. plicatilis* and *A. franciscana* to first feeding conditions resulted in a decrease in the total bacterial numbers, but the added bacteria remained a significant part of their bacterial flora (Paper 4). Thus, the two live food organisms showed potential as vectors for probiotic bacteria to marine fish larvae.
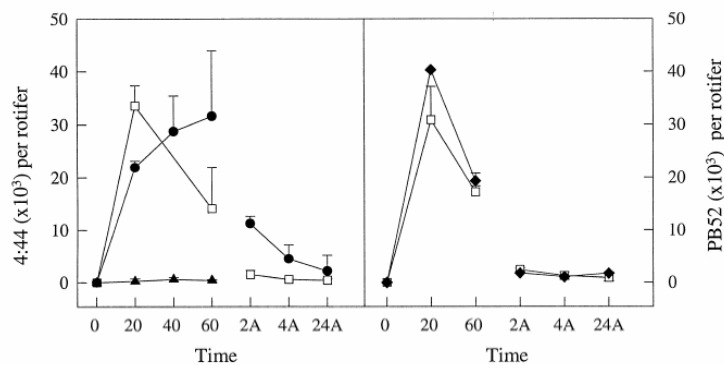


**Figure 11.** The number of added bacteria in rotifers after grazing in bacterial suspensions (0–60 min) and transfer to first feeding conditions (2 - 24 hours). The added bacteria were detected by ICB. The concentrations in bacterial suspensions were $7.8 \times 10^6$ 4:44 ml$^{-1}$ (▲), $1.7 \times 10^8$ 4:44 ml$^{-1}$ (●), mixture of 4:44 and PB52 ($3.5 \times 10^8$ bacteria ml$^{-1}$, □), and $3.3 \times 10^8$ PB52 ml$^{-1}$ (♦).

Rotifers were used as live feed and for bioencapsulation of bacteria, in the first feeding experiments with turbot (Paper 5) and cod larvae (Paper 6). Bacterial suspensions with a density of $10^8$ bacteria ml$^{-1}$ were used during bioencapsulation. In the first feeding experiment with turbot larvae (Paper 5), ICB and ELISA were used to detect the added bacteria, whereas T-RFLP was used in cod experiment (Paper 6). After the bioencapsulation procedure, the added bacteria constituted a considerably lower proportion of the flora of the rotifers when analysed with T-RFLP (Paper 6), compared to rotifers that were analysed by ICB (Paper 4 and 5). This discrepancy could be caused by (1) the different detection methods used, i.e. culture based vs. culture independent method; (2) a more thorough washing procedure was

used in the cod experiment, which might remove more of the surface associated bacteria (Munro et al. , 1993); (3) different bacteria were used, and differences have been observed in the ability of bacteria to establish in rotifers (Paper 4).

## First feeding experiments with marine larvae

Large numbers of bacterial isolates can be effectively screened and evaluated based on *in vitro* properties. However, even if the isolates perform well in the *in vitro* tests, they must have beneficial effects under *in vivo* conditions to be regarded as probiotics (Gram and Ringø, 2005). *In vivo* experiments are also important to verify that fish larvae are in fact colonised by the added probiotic candidates. In this thesis, two methods to obtain controlled bacterial colonization of fish larvae were tested in a first feeding experiment with turbot larvae (Paper 5). In addition, probiotic candidates from the dominant and from the antagonistic bacterial flora of healthy cod larvae were evaluated under *in vivo* conditions in a first feeding experiment with cod larvae (Paper 6).

To evaluate methods for controlled colonization of the larval gut in a first feeding experiment with turbot larvae, the bacteria were 1) added directly into the rearing water on the day of hatching, 2) bioencapsulated in rotifers on day 2 after hatching, or 3) administered by both previous methods (Paper 5). The larvae were reared with stagnant rearing water until day 6 after hatching. The method of addition did not influence the numbers of added bacteria in the rearing water on day 3 after hatching, or the colonization of the larvae by the added bacteria. Both bacterial strains (4:44 and PB52) colonized the gut of turbot larvae. However, isolate PB52 colonized larvae at considerably higher numbers than 4:44. Thus, in this thesis and a similar study (Ringø and Vadstein, 1998), it was shown that different bacteria have different colonization potential in turbot larvae. In addition, it was found that one of the isolates colonized the larvae more efficiently when added in a mixture with the other strain, whereas the opposite was found for the other bacterial isolate. This was different from what was found when *Vibrio pelagius* and *Aeromonas caviae* were added to the rearing water of turbot larvae (Ringø and Vadstein, 1998), where both species of bacteria colonized fish larvae at lower levels when added in a mixture, compared to when they were added alone. Thus, probiotic mixtures of bacteria can have different colonization potential than the individual strains in the mixture would have alone.

The probiotic candidates that were used in the first feeding experiment with Atlantic cod larvae (Paper 6) were selected from the dominant (Paper 1) and from the antagonistic (Paper 2) bacterial flora of healthy cod larvae. The bacterial isolates were chosen based on their *in vitro* probiotic properties, and finally on performance in the small-scale *in vivo* test with cod larvae (Paper 3). From the dominant bacterial flora one *Vibrio* (RD5-30) and two *Microbacterium* strains (ID3-10 and ND2-7) were selected, whereas from the antagonistic bacterial flora one *Roseobacter* (RA4-1) and one *Pseudoalteromonas* (RA7-14) were selected (for properties, see Table 1). Three treatments were tested; 1. Control with no addition, 2. Addition of dominant bacteria, 3. Addition of antagonistic bacteria. The probiotic candidates were added both into the rearing water on the day of hatching and bioencapsulated in rotifers on several days (day 2, 3, 4, 6, 8, 11 and 16). Both methods of addition were used because the cod larvae were reared with water exchange from day 0, and thus the added bacteria were constantly washed out of the rearing system. Elevated levels, however not significantly elevated, of added bacteria were detected in water and rotifers after the addition. However, the added bacteria did not colonize the rearing water, the rotifers or the cod larvae at detectable levels over time. Thus, the colonization potential of the added bacteria was apparently poor in this experiment. The added isolate RA4-1 had considerably better *in vitro* adhesive ability to mucus, compared to the other added bacteria. This was, however, not reflected in the *in vivo* colonization of cod larvae. Pooled samples of 10 cod larvae were analyzed with T-RFLP on all samplings, however, on day 17 also individual larvae were analyzed. The T-RFLP data from the individual larvae showed variable levels of TRFs corresponding to the added and also for other bacteria, implying that different bacteria colonized different individuals in varying numbers (Figure 12). This is in accordance with inter-individual variation found in the bacterial flora of cod larvae (Paper 1, Paper 2), as well as in pigs (Simpson et al. , 2000) and broiler chickens (Zhu et al. , 2002). This profound inter-individual variation adds complexity to the microbial management of marine larviculture. Neither the growth nor the survival of cod larvae was different in treated tanks, compared to the control. Hence, the added bacteria apparently did not improve the vitality of larvae. However, both treated tanks and control tanks had high growth rates in the rotifer feeding period, compared to previous studies (Browman et al. , 2006; Park et al. , 2006). Moreover, in a first feeding experiment  performed in the same rearing system as the present study (Skjermo et al. 2006), the dryweight of cod larvae was ± 250 µg on day 17, compared to 497 to 701 µg on day 17 in the present study. A relatively high survival of 41.5 - 80.1% was obtained in larvae in the weaning phase in the present study, compared to Skjermo et al. (2006). Thus, both growth rate and survival indicate

high viability for both treated and control larvae. This implies that the potential for further improvement might have been low. Probiotics can also improve stress tolerance and non-specific immune responses in fish (Taoka et al. , 2006). The transition from live feed to dry feed was performed between day 18 and day 21 in the present study. This is an early transfer to dry feed, and was assumed to work as a stress test. The growth rates of the cod larvae were severely hampered by the transition to dry feed (from 20-30 %SGR day$^{-1}$ before to 2-6 %SGR day$^{-1}$ after transition), however, a relatively high survival was obtained in the weaning phase. Thus, based on growth and mortality of larvae, there were no differences in stress tolerance between treated and control larvae.
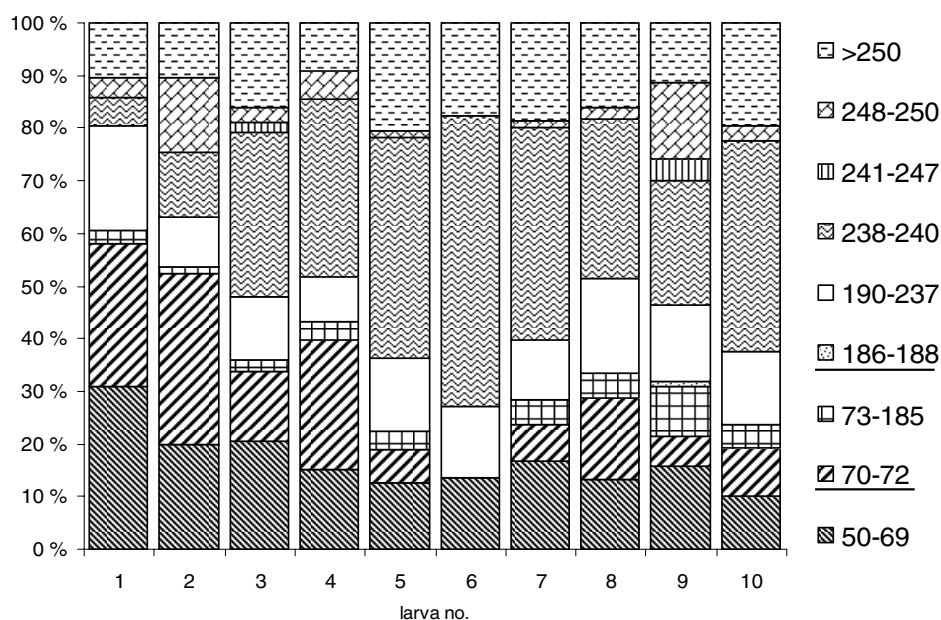


**Figure 12.** The percentage of terminal restriction fragments obtained from 10 individual larvae on day 17 after hatching in the first feeding experiment with cod larvae for treatment D (added the dominant bacteria *Microbacterium*, TRF 71 ± 1 bp, and *Vibrio*, TRF 187 ± 1 bp).

## Conclusions

The dominant intestinal bacterial flora in cod larvae varied both qualitatively and quantitatively between individual larvae and between rearing groups. The bacterial flora of larvae was dominated by the Gram positive *Microbacterium* and by Gram negative bacteria within the γ-Proteobacteria.

The antagonistic intestinal bacterial flora in cod larvae varied in composition between individual larvae both within and between different rearing groups. In addition, the percentage of antagonistic bacteria in larvae varied within and between larvae from different rearing groups. The majority of antagonistic bacteria belonged to γ-Proteobacteria, but also members of α-Proteobacteria were found to be antagonistic to *L. anguillarum*.

The *in vitro* tests on antagonism, adhesion to mucus, growth in mucus, production of extracellular enzymes, bile resistance and haemolytic activity were efficient in screening many bacterial isolates for properties considered as important for probiotic bacteria. The test results showed large differences between the bacterial isolates. The experimental exposure test of yolk sac cod larvae with candidate probiotic bacteria revealed bacterial strains that were detrimental to the larvae. The test also revealed that five candidate bacteria gave improved survival of cod larvae, compared to the control.

Bioencapsulation of bacteria in live feed was achieved after short incubations (20 - 60 min) in bacterial suspensions. Bioencapsulation was most efficient and predictable when using high density bacterial suspensions ($10^8$ bacteria ml$^{-1}$). A decrease in the bioencapsulated bacteria occurred after transfer to first feeding conditions. However, the bacteria were still present in the live feed 24 hours after transfer. The bioencapsulation efficiency varied between bacterial strains, and whether the bacteria were added alone or in mixture. A lower amount of the bacterial flora of rotifers seemed to be exchanged when analyzed by T-RFLP, compared to experiments analyzed by ICB. Both bacterial species differences and differences between the detection methods should be further investigated to assure controlled colonization of the live feed.

Controlled colonization of turbot larvae was obtained with bacteria added either in the water or bioencapsulated in rotifers, whereas probiotic candidates that were added to a cod rearing

system did not persist at detectable levels in water, rotifers or larvae. These differences could have several reasons; the two different fish species might respond differently to addition of bacteria, different bacteria were added in the two experiments, different methods were used to detect the added bacteria, and the turbot rearing was stagnant for 6 days whereas the cod rearing had water exchange from day 0. Controlled colonization of the host is crucial when adding probiotic bacteria. Our results suggest that the techniques for transfer of bacteria might not be directly transferable between different rearing regimes and between different fish and bacterial species.

The probiotic candidates that were added in the cod larvae experiment were selected by two screening strategies, i.e. as part of the dominant or the antagonistic intestinal bacterial flora of healthy cod larvae. Based on the results obtained from the *in vitro* and *in vivo* experiments, one of the selection strategies can not be recommended over the other.

## Future perspectives

Understanding the mechanisms of probiotic action, and obtaining further knowledge of the microbial ecology in the gut of fish larvae is essential in order to select probiotic bacteria that will have beneficial effects on the host *in vivo*. To achieve this, more studies on host-bacterial interactions are needed, and the methods in molecular biology will undoubtedly be of great importance. Methods to characterize microbial communities efficiently (e.g. DGGE, T-RFLP), and methods to detect and quantify specific bacteria (e.g. real time PCR with Taqman probes) should be used to better understand the competition between bacteria in the gut. Microarray techniques can be used to detect how the bacterial flora influences gene expression in the host and different bacteria have already been shown to influence gene expression in zebrafish differently (Rawls et al. , 2004). This can be a powerful method to understand some of the host-bacteria interactions. In addition, bacterial quorum sensing might be important in determining the species composition in microbial communities. Recently, disruption of bacterial quorum sensing has been proposed as a strategy against infections in aquaculture (Defoirdt et al. , 2004).

It is important to select between single- and multiple- strain probiotics based on rational criteria. Addition of several bacteria with supplementary properties might have greater probiotic potential than addition of single strains, and synergistic effects can be the result when using mixtures of bacteria. The individual differences in bacterial flora composition

between cod larvae, suggests that in a mixture of several bacterial isolates the probability that at least one isolate colonizes the larvae is greater. However, if probiotics are added to protect against a specific pathogen, the addition of single - strain probiotics might be relevant.

The acquisition of a pool of bacterial isolates is the first step towards finding probiotic bacteria. Different selection strategies can be used in this initial phase to obtain the best possible pool of isolates. In this thesis, the initial selection strategies were selecting dominant bacteria and selecting antagonistic bacteria. Also other criteria have been used in the initial screening phase, such as screening for isolates with high adhesiveness to mucus. Even though it is laborious, a combination of methods is probably the best way to go, especially if a mixture of probiotic bacteria with supplementary properties is the final goal.

*In vitro* tests of bacterial properties have to be used to lower the number of candidate bacteria. Today, bacterial properties on antagonism and mucus adhesion are mostly used. In the future, further studies on the nutritive contribution of the intestinal bacterial flora, the antiviral activity, quorum sensing activity and the influence on host gene expression should be conducted.

Challenge tests with pathogenic bacteria at the end of first feeding experiments can show whether the addition of probiotic bacteria cause protection against infection. The use of challenge tests might also help explain the mechanisms of probiotic action.

Non-digestible food ingredients that alter the composition, or metabolism, of the gut bacterial flora in a beneficial manner (prebiotics) are also gaining interest. Prebiotics are cheaper and carry less risk than probiotics. Prebiotics can be added alone, or in combination with probiotics (synbiotics). The concept of synbiotics can assist the colonization of the host with added probiotics, by supplying the probiotic bacteria with nutrition.

The ongoing arms race between the use of antibiotics and antibiotics resistance in bacteria has already urged the need for alternative strategies in the fight against disease in animal husbandry and in aquaculture. The use of probiotics may be one of several measures to solve the problems. Extensive research on the mechanisms of action of the bacteria has to be performed to obtain efficient probiotics for all species and all conditions. Other aspects of probiotics that have to be regarded are the safety (Anadon et al. , 2006; Silley, 2006) and

patenting regulations that make the way towards commercially ready products long and expensive.

## References

Adams A. (1991) Detection of *Vibrio parahaemolyticus* biotype alginolyticus in penaeid shrimps using an amplified enzyme-linked-immunosorbent-assay. Aquaculture 93:101-108.

Anadon A., Martinez-Larranaga M.R., Martinez M.A. (2006) Probiotics for animal nutrition in the European Union. Regulation and safety assessment. Regul. Toxicol. Pharmacol. 45:91-95.

Atlas R.M. (1999) Probiotics - snake oil for the new millennium? Environ. Microbiol. 1:377-382.

Attramadal K.J.K. (2004) Water quality and microbial environment in a flow through and a recirculating system for intensive rearing of cod larvae (*Gadhus morhua* L.). Master thesis in Aquaculture and Marine Resources. Norwegian University of Science and Technology (NTNU).

Austin B., Austin D., Sutherland R., Thompson F., Swings J. (2005) Pathogenicity of vibrios to rainbow trout (*Oncorhynchus mykiss*, Walbaum) and *Artemia* nauplii. Environ. Microbiol. 7:1488-1495.

Bagge-Ravn D., Ng Y., Hjelm M., Christiansen J.N., Johansen C., Gram L. (2003) The microbial ecology of processing equipment in different fish industries - analysis of the microflora during processing and following cleaning and disinfection. Int. J. Food Microbiol. 87:239-250.

Bairagi A., Ghosh K.S., Kumar S., Sen S.K., Ray A.K. (2002) Enzyme producing bacterial flora isolated from fish digestive tracts. Aquacult. Int. 10:109-121.

Balcazar J.L., de Blas I., Ruiz-Zarzuela I., Cunningham D., Vendrell D., Muzquiz J.L. (2006) The role of probiotics in aquaculture. Vet. Microbiol. 114:173-186.

Bergh Ø., Hansen G.H., Taxt R.E. (1992) Experimental-infection of eggs and yolk-sac larvae of halibut, *Hippoglossus hippoglossus* L. J. Fish Dis. 15:379-391.

Bizani D., Brandelli A. (2004) Influence of media and temperature on bacteriocin production by *Bacillus cereus* 8A during batch cultivation. Appl. Microbiol. Biotechnol. 65:158-162.

Blanch A.R., Alsina M., Simon M., Jofre J. (1997) Determination of bacteria associated with reared turbot (*Scophthalmus maximus*) larvae. J. Appl. Microbiol. 82:729-734.

Browman H.I., Skiftesvik A.B., Kuhn P. (2006) The relationship between ultraviolet and polarized light and growth rate in the early larval stages of turbot (*Scophtalmus maximus*), Atlantic cod (*Gadus morhua*) and Atlantic herring (*Clupea harengus*) reared in intensive culture conditions. Aquaculture 256:296-301.

Bruhn J.B., Haagensen J.A.J., Bagge-Ravn D., Gram L. (2006) Culture conditions of *Roseobacter* strain 27-4 affect its attachment and biofilm formation as quantified by real-time PCR. Appl. Environ. Microbiol. 72:3011-3015.

Chabrillon M., Rico R.M., Balebona M.C., Morinigo M.A. (2005) Adhesion to sole, *Solea senegalensis* Kaup, mucus of microorganisms isolated from farmed fish, and their interaction with *Photobacterium damselae* subsp *piscicida*. J. Fish Dis. 28:229-237.

Conceicao L.E.C., Skjermo J., Skjåk-Bræk G., Verreth J.A.J. (2001) Effect of an immunostimulating alginate on protein turnover of turbot (*Scophthalmus maximus* L.) larvae. Fish Physiol. Biochem. 24:207-212.

De Schrijver R., Ollevier F. (2000) Protein digestion in juvenile turbot (*Scophthalmus maximus*) and effects of dietary administration of *Vibrio proteolyticus*. Aquaculture 186:107-116.

Defoirdt T., Boon N., Bossier P., Verstraete W. (2004) Disruption of bacterial quorum sensing: an unexplored strategy to fight infections in aquaculture. Aquaculture 240:69-88.

Dumonceaux T.J., Hill J.E., Briggs S.A., Amoako K.K., Hemmingsen S.M., Van Kessel A.G. (2006) Enumeration of specific bacterial populations in complex intestinal communities using quantitative PCR based on the chaperonin-60 target. J. Microbiol. Methods 64:46-62.

Eddy S.D., Jones S.H. (2002) Microbiology of summer flounder *Paralichthys dentatus* fingerling production at a marine fish hatchery. Aquaculture 211:9-28.

Fuller R. (1989) Probiotics in man and animals. J. Appl. Bacteriol. 66:365-378.

Gatesoupe F.J. (1994) Lactic-acid bacteria increase the resistance of turbot larvae, *Scophthalmus maximus*, against pathogenic *vibrio*. Aquat. Living Res. 7:277-282.

Gatesoupe F.J. (1999) The use of probiotics in aquaculture. Aquaculture 180:147-165.

Gill S.R., Pop M., DeBoy R.T., Eckburg P.B., Turnbaugh P.J., Samuel B.S., Gordon J.I., Relman D.A., Fraser-Liggett C.M., Nelson K.E. (2006) Metagenomic analysis of the human distal gut microbiome. Science 312:1355-1359.

Gram L., Ringø E. (2005) Prospects of fish probiotics. In: Holzapfel W., Naughton P. (eds) Microbial ecology of growing animals. Elsevier.

Grossart H.P., Schlingloff A., Bernhard M., Simon M., Brinkhoff T. (2004) Antagonistic activity of bacteria isolated from organic aggregates of the German Wadden Sea. FEMS Microbiol. Ecol. 47:387-396.

Hansen G.H., Olafsen J.A. (1999) Bacterial interactions in early life stages of marine cold water fish. Microbial Ecol. 38:1-26.

Hansen G.H., Sørheim R. (1991) Improved method for phenotypical characterization of marine-bacteria. J. Microbiol. Methods 13:231-241.

Hansen G.H., Strøm E., Olafsen J.A. (1992) Effect of different holding regimens on the intestinal microflora of herring (*Clupea harengus*) larvae. Appl. Environ. Microbiol. 58:461-470.

Hjelm M., Bergh Ø., Riaza A., Nielsen J., Melchiorsen J., Jensen S., Duncan H., Ahrens P., Birkbeck T.H., Gram L. (2004a) Selection and identification of autochthonous potential probiotic bacteria from turbot larvae (*Scophthalmus maximus*) rearing units. Syst. Appl. Microbiol. 27:360-371.

Hjelm M., Riaza A., Formoso F., Melchiorsen J., Gram L. (2004b) Seasonal incidence of autochthonous antagonistic *Roseobacter* spp. and *Vibrionaceae* strains in a turbot larva (*Scophthalmus maximus*) rearing system. Appl. Environ. Microbiol. 70:7288-7294.

Hoehne-Reitan K., Kjørsvik E., Reitan K.I. (2001) Development of the pH in the intestinal tract of larval turbot. Mar. Biol. 139:1159-1164.

Holmström C., Egan S., Franks A., McCloy S., Kjelleberg S. (2002) Antifouling activities expressed by marine surface associated *Pseudoalteromonas* species. FEMS Microbiol. Ecol. 41:47-58.

Hopkins M.J., Sharp R., Macfarlane G.T. (2002) Variation in human intestinal microbiota with age. Dig. Liver Dis. 34:S12-S18.

Huys L., Dhert P., Robles R., Ollevier F., Sorgeloos P., Swings J. (2001) Search for beneficial bacterial strains for turbot (*Scophthalmus maximus* L.) larviculture. Aquaculture 193:25-37.

Irianto A., Austin B. (2002) Probiotics in aquaculture. J. Fish Dis. 25:633-642.

Jernberg C., Sullivan A., Edlund C., Jansson J.K. (2005) Monitoring of antibiotic-induced alterations in the human intestinal microflora and detection of probiotic strains by use of terminal restriction fragment length polymorphism. Appl. Environ. Microbiol. 71:501-506.

Jung S.Y., Lee J.H., Chai Y.G., Kim S.J. (2005) Monitoring of microorganisms added into oil-contaminated microenvironments by terminal-restriction fragment length polymorphism analysis. J. Microbiol. Biotechnol. 15:1170-1177.

Kanagawa T. (2003) Bias and artifacts in multitemplate polymerase chain reactions (PCR). J. Biosci. Bioeng. 96:317-323.

Kaplan C.W., Kitts C.L. (2003) Variation between observed and true Terminal Restriction Fragment length is dependent on true TRF length and purine content. J. Microbiol. Methods 54:121-125.

Korsnes K., Nicolaisen O., Skar C.K., Nerland A.H., Bergh Ø. (2006) Bacteria in the gut of juvenile cod *Gadus morhua* fed live feed enriched with four different commercial diets. ICES J. Mar. Sci. 63:296-301.

Kotani H., Mcgarrity G.J. (1986) Identification of mycoplasma colonies by immunobinding. J. Clin. Microbiol. 23:783-785.

Liu W.T., Marsh T.L., Cheng H., Forney L.J. (1997) Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. Appl. Environ. Microbiol. 63:4516-4522.

Long R.A., Azam F. (2001) Antagonistic interactions among marine pelagic bacteria. Appl. Environ. Microbiol. 67:4975-4983.

Makridis P., Martins S., Tsalavouta M., Dionisio L.C., Kotoulas G., Magoulas A., Dinis M.T. (2005a) Antimicrobial activity in bacteria isolated from Senegalese sole, *Solea senegalensis*, fed with natural prey. Aquacult. Res. 36:1619-1627.

Makridis P., Martins S., Vercauteren T., Van Driessche K., Decamp O., Dinis M.T. (2005b) Evaluation of candidate probiotic strains for gilthead sea bream larvae (*Sparus aurata*) using an in vivo approach. Lett. Appl. Microbiol. 40:274-277.

Makridis P., Vadstein O. (1999) Food size selectivity of *Artemia franciscana* at three developmental stages. J. Plankton Res. 21:2191-2201.

Mangor Jensen A., Adoff G.R. (1987) Drinking activity of the newly hatched larvae of cod *Gadus morhua* L. Fish Physiol. Biochem. 3:99-103.

Monteiro L., Mariano R.D.R., Souto-Maior A.M. (2005) Antagonism of *Bacillus* spp. against *Xanthomonas campestris* pv. *campestris*. Braz. Arch. Biol. Technol. 48:23-29.

Munn C.B. (1978) Hemolysin production by *Vibrio anguillarum*. FEMS Microbiol. Lett. 3:265-268.

Munro P.D., Birkbeck T.H., Barbour A. (1993) Bacterial flora of rotifers (*Brachionus plicatilis*): Evidence for a major location on the external surface and methods for

reducing the rotifer bacterial load. In: Reinertsen, Dahle, Jørgensen, Tvinnerheim (eds) Fish farming technology. Balkema, Rotterdam, pp 93-100.

Munro P.D., Henderson R.J., Barbour A., Birbeck T.H. (1999) Partial decontamination of rotifers with ultraviolet radiation: the effect of changes in the bacterial load and flora of rotifers on mortalities in start-feeding larval turbot. Aquaculture 170:229-244.

Muroga K., Higashi M., Keitoku H. (1987) The isolation of intestinal microflora of farmed red seabream (*Pagrus major*) and black seabream (*Acanthopagrus schlegeli*) at larval and juvenile stages. Aquaculture 65:79-88.

Nedoluha P.C., Westhoff D. (1997) Microbiology of striped bass grown in three aquaculture systems. Food Microbiol. 14:255-264.

Nikoskelainen S., Salminen S., Bylund G., Ouwehand A.C. (2001) Characterization of the properties of human- and dairy-derived probiotics for prevention of infectious diseases in fish. Appl. Environ. Microbiol. 67:2430-2435.

Olafsen J.A. (2001) Interactions between fish larvae and bacteria in marine aquaculture. Aquaculture 200:223-247.

Olsen A.I., Olsen Y., Attramadal Y., Christie K., Birkbeck T.H., Skjermo J., Vadstein O. (2000) Effects of short term feeding of microalgae on the bacterial flora associated with juvenile *Artemia franciscana*. Aquaculture 190:11-25.

Olsson J.C., Westerdahl A., Conway P.L., Kjelleberg S. (1992) Intestinal colonization potential of turbot (*Scophthalmus maximus*)- and dab (*Limanda limanda*)-associated bacteria with inhibitory effects against *Vibrio anguillarum*. Appl. Environ. Microbiol. 58:551-556.

Osborn A.M., Moore E.R.B., Timmis K.N. (2000) An evaluation of terminal-restriction fragment length polymorphism (T-RFLP) analysis for the study of microbial community structure and dynamics. Environ. Microbiol. 2:39-50.

Osborne C.A., Rees G.N., Bernstein Y., Janssen P.H. (2006) New threshold and confidence estimates for terminal restriction fragment length polymorphism analysis of complex bacterial communities. Appl. Environ. Microbiol. 72:1270-1278.

Park H.G., Puvanendran V., Kellett A., Parrish C.C., Brown J.A. (2006) Effect of enriched rotifers on growth, survival, and composition of larval Atlantic cod (*Gadus morhua*). ICES J. Mar. Sci. 63:285-295.

Perez-Casanova J.C., Murray H.M., Gallant J.W., Ross N.W., Douglas S.E., Johnson S.C. (2006) Development of the digestive capacity in larvae of haddock (*Melanogrammus aeglefinus*) and Atlantic cod (*Gadus morhua*). Aquaculture 251:377-401.

Planas M., Perez-Lorenzo M., Hjelm M., Gram L., Fiksdal I.U., Bergh Ø., Pintado J. (2006) Probiotic effect in vivo of *Roseobacter* strain 27-4 against *Vibrio* (*Listonella*) *anguillarum* infections in turbot (*Scophthalmus maximus* L.) larvae. Aquaculture 255:323-333.

Ramirez R.F., Dixon B.A. (2003) Enzyme production by obligate intestinal anaerobic bacteria isolated from oscars, (*Astronotus ocellatus*), angelfish (*Pterophyllum scalare*) and southern flounder (*Paralichthys lethostigma*). Aquaculture 227:417-426.

Rawls J.F., Samuel B.S., Gordon J.I. (2004) Gnotobiotic zebrafish reveal evolutionarily conserved responses to the gut microbiota. Proc. Natl. Acad. Sci. U S A 101:4596-4601.

Reitan K.I., Natvik C.M., Vadstein O. (1998) Drinking rate, uptake of bacteria and microalgae in turbot larvae. J. Fish Biol. 53:1145-1154.

Ringø E., Birkbeck T.H. (1999) Intestinal microflora of fish larvae and fry. Aquacult. Res. 30:73-93.

Ringø E., Birkbeck T.H., Munro P.D., Vadstein O., Hjelmeland K. (1996) The effect of early exposure to *Vibrio pelagius* on the aerobic bacterial flora of turbot, *Scophthalmus maximus* (L) larvae. J. Appl. Bacteriol. 81:207-211.

Ringø E., Gatesoupe F.J. (1998) Lactic acid bacteria in fish: a review. Aquaculture 160:177-203.

Ringø E., Vadstein O. (1998) Colonization of *Vibrio pelagius* and *Aeromonas caviae* in early developing turbot (*Scophthalmus maximus* L.) larvae. J. Appl. Microbiol. 84:227-233.

Rowland I.R. (1992) Metabolic interactions in the gut. In: Fuller R. (ed) Probiotics. The scientific basis. Chapman & Hall, London, pp 29-54.

Salvesen I., Skjermo J., Vadstein O. (1999) Growth of turbot (*Scophthalmus maximus* L.) during first feeding in relation to the proportion of *r/K*-strategists in the bacterial community of the rearing water. Aquaculture 175:337-350.

Silley P. (2006) Do bacteria need to be regulated? J. Appl. Microbiol. 101:607-615.

Simpson J.M., McCracken V.J., Gaskins H.R., Mackie R.I. (2000) Denaturing gradient gel electrophoresis analysis of 16S ribosomal DNA amplicons to monitor changes in fecal bacterial populations of weaning pigs after introduction of *Lactobacillus reuteri* strain MM53. Appl. Environ. Microbiol. 66:4705-4714.

Skjermo J., Bergh Ø. (2004) High-M alginate immunostimulation of Atlantic halibut (*Hippoglossus hippoglossus* L.) larvae using *Artemia* for delivery, increases resistance against vibriosis. Aquaculture 238:107-113.

Skjermo J., Salvesen I., Øie G., Olsen Y., Vadstein O. (1997) Microbially matured water: A technique for selection of a non-opportunistic bacterial flora in water that may improve performance of marine larvae. Aqucult. Int. 5:13-28.

Skjermo, J., Størseth, T.R., Hansen, K., Handå, A., Øie, G. (2006) Evaluation of β-(1→3, 1→6)-glucans and High-M alginate used as immunostimulatory dietary supplement during first feeding and weaning of Atlantic cod (*Gadhus morhua* L.). Aquaculture, accepted.

Skjermo J., Vadstein O. (1999) Techniques for microbial control in the intensive rearing of marine larvae. Aquaculture 177:333-343.

Spanggaard B., Huber I., Nielsen J., Nielsen T., Appel K.F., Gram L. (2000) The microflora of rainbow trout intestine: a comparison of traditional and molecular identification. Aquaculture 182:1-15.

Sugita H., Kurosaki M., Okamura T., Yamamoto S., Tsuchiya C. (2005) The culturability of intestinal bacteria of Japanese coastal fish. Fish. Sci. 71:956-958.

Sugita H., Okano R., Suzuki Y., Iwai S., Mizukami M., Akiyama N., Matsuura S. (2002) Antibacterial abilities of intestinal bacteria from larval and juvenile Japanese flounder against fish pathogens. Fish. Sci. 68:1004-1011.

Sugita H., Shibuya K., Shimooka H., Deguchi Y. (1996) Antibacterial abilities of intestinal bacteria in freshwater cultured fish. Aquaculture 145:195-203.

Suzuki M.T., Giovannoni S.J. (1996) Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. Appl. Environ. Microbiol. 62:625-630.

Taoka Y., Maeda H., Jo J.Y., Jeon M.J., Bai S.C., Lee W.J., Yuge K., Koshio S. (2006) Growth, stress tolerance and non-specific immune response of Japanese flounder *Paralichthys olivaceus* to probiotics in a closed recirculating system. Fish. Sci. 72:310-321.

Vaahtovuo J., Toivanen P., Eerola E. (2003) Bacterial composition of murine fecal microflora is indigenous and genetically guided. FEMS Microbiol. Ecol. 44:131-136.

Vadstein O. (1997) The use of immunostimulation in marine larviculture: possibilities and challenges. Aquaculture 155:401-417.

Vadstein O., Mo T.A., Bergh Ø. (2004) Microbial interactions, prophylaxis and diseases. In: Moksness E., Kjørsvik E., Olsen Y. (eds) Culture of cold-water marine fish. Blackwell Publishing Ltd, pp 28-72.

Vadstein O., Øie G., Olsen Y. (1993) Particle-size dependent feeding by the rotifer *Brachionus plicatilis*. Hydrobiol. 255:261-267.

Verner-Jeffreys D.W., Shields R.J., Bricknell I.R., Birkbeck T.H. (2003) Changes in the gut-associated microflora during the development of Atlantic halibut (*Hippoglossus hippoglossus* L.) larvae in three British hatcheries. Aquaculture 219:21-42.

Verschuere L., Dhont J., Sorgeloos P., Verstraete W. (1997) Monitoring Biolog patterns and *r/K*-strategists in the intensive culture of *Artemia* juveniles. J. Appl. Microbiol. 83:603-612.

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

Vine N.G., Leukes W.D., Kaiser H. (2004a) *In vitro* growth characteristics of five candidate aquaculture probiotics and two fish pathogens grown in fish intestinal mucus. FEMS Microbiol. Lett. 231:145-152.

Vine N.G., Leukes W.D., Kaiser H. (2006) Probiotics in marine larviculture. FEMS Microbiol. Rev. 30:404-427.

Vine N.G., Leukes W.D., Kaiser H., Daya S., Baxter J., Hecht T. (2004b) Competition for attachment of aquaculture candidate probiotic and pathogenic bacteria on fish intestinal mucus. J. Fish Dis. 27:319-326.

Westerdahl A., Olsson J.C., Kjelleberg S., Conway P.L. (1991) Isolation and characterization of turbot (*Scophthalmus maximus*)-associated bacteria with inhibitory effects against *Vibrio anguillarum*. Appl. Environ. Microbiol. 57:2223-2228.

Zhu X.Y., Zhong T.Y., Pandya Y., Joerger R.D. (2002) 16S rRNA-based analysis of microbiota from the cecum of broiler chickens. Appl. Environ. Microbiol. 68:124-137.

Zoetendal E.G., Akkermans A.D.L., De Vos W.M. (1998) Temperature gradient gel electrophoresis analysis of 16S rRNA from human fecal samples reveals stable and host-specific communities of active bacteria. Appl. Environ. Microbiol. 64:3854-3859.

Zoetendal E.G., Collier C.T., Koike S., Mackie R.I., Gaskins H.R. (2004) Molecular ecological analysis of the gastrointestinal microbiota: A review. J. Nutr. 134:465-472.

# Paper I

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# Paper II

**The antagonistic bacterial flora of intensively reared cod larvae is dominated by**

**<u>Vibrio</u>**

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**Abstract**

The intestinal bacterial flora in 27 Atlantic cod larvae from five rearing groups was screened for antagonistic activity against <u>Listonella anguillarum</u>. A total of 98 antagonistic isolates were phenotypically characterized and belonged to 12 clusters at the 80% similarity level. The 16S rDNA of representative isolates was partially sequenced and compared to sequences in the GenBank database. The majority of antagonistic bacterial isolates had high sequence similarity to γ-Proteobacteria, and the rest were members of α-Proteobacteria. Antagonistic isolates with sequence similarity to <u>Vibrio</u> were found in larvae from all five rearing groups, and was the most numerous group of antagonistic isolates. The other antagonistic bacteria had sequence similarities to <u>Hyphomicrobium, Marinomonas, Pseudoalteromonas, Roseobacter</u> and <u>Shewanella</u>. The composition of the antagonistic bacterial flora varied both between individual cod larvae from the same rearing group and between larvae from the five different rearing groups. Also, the average percentage of antagonistic isolates was significantly different in one of the five rearing groups.

**Keywords**

**Introduction**

The composition of the bacterial flora in the intestinal tract is a result of several selecting mechanisms, such as competition for resources (nutrients and space) and chemical warfare through production of compounds that are inhibitory for other bacteria. In vitro antagonism against one or several bacteria is widely distributed among marine pelagic bacteria (Long and Azam, 2001; Grossart et al. , 2004) and in bacteria isolated from marine fish larvae (Westerdahl et al. , 1991; Sugita et al. , 2002; Makridis et al. , 2005). Antagonistic compounds are defined as chemical substances that are produced by bacteria and that are toxic or inhibitory towards other microorganisms. They include primary and secondary metabolites such as antibiotics, organic acids, hydrogen peroxide, siderophores and bacteriocins.

Several bacterial genera produce antibacterial compounds (Sugita et al. , 1996; Ringø and Gatesoupe, 1998; Long and Azam, 2001; Hjelm et al. , 2004b; Makridis et al. , 2005). Generally, only a few percent of the cultivable bacterial flora from fish shows inhibition of pathogenic bacteria in vitro (Sugita et al. , 1996, 2002; Hjelm et al. , 2004a), but higher percentages have also been reported (Westerdahl et al. , 1991). In Senegalese sole the percentage of antagonistic bacteria in the gut bacterial flora increased when the fish were fed natural prey, and after six weeks almost 40% of the intestinal bacteria inhibited Listonella anguillarum and Photobacterium damselae (Makridis et al. , 2005).

The intestinal bacterial flora is important in the prevention of colonization by pathogens (Hentges, 1992). The reasons for this protection are not fully understood, but

competitive exclusion is a likely factor. Another possible reason for the protective role of the intestinal bacterial flora is that bacteria in the gut produce compounds that are growth-inhibiting to pathogens. Although antagonism between bacteria is thought to be an important factor in the defence against pathogens, it is not known if antagonistic activity in an in vitro assay has any effect in vivo (Atlas, 1999). In the study performed by Westerdahl et al. (1991) it was shown that a significantly higher proportion of intestinal rather than surface associated bacteria displayed inhibitory effects against L. anguillarum in turbot. This suggests that bacteria with inhibitory abilities were favoured in the intestinal tract.

Over the past years the interest in intensive aquaculture production of Atlantic cod (Gadhus morhua L.) has increased. However, the production of cod larvae has been problematic owing to low and unpredictable growth and survival during first-feeding. Marine larvae experience stress due to high larval densities, and are in constant interaction with bacteria during first feeding (Olafsen, 2001; Vadstein et al. , 2004). The rearing conditions may enhance the proliferation of opportunistic bacteria, and these bacteria can be detrimental to the larvae (Skjermo et al. , 1997). Thus, microbial problems are important reasons for the high and unpredictable growth and survival that is experienced. The use of probiotic bacteria is one of several methods that have been suggested to obtain microbial control in the intensive rearing of marine larvae (Skjermo and Vadstein, 1999). Screening for antagonistic bacteria is a widely used method in the search for probiotic bacteria (Gram and Ringø, 2005). In this study, we isolated and phenotypically characterized the cultivable antagonistic intestinal bacterial flora of Atlantic cod larvae reared under variable conditions. Furthermore, a selection of the

antagonistic isolates were characterized by 16S rDNA phylogenetic analysis. The antagonistic activity was tested in vitro against L. anguillarum (strain HI610). The antagonistic bacterial isolates will serve as a pool of probiotics candidates for later evaluation.

**Materials and methods**

*Larval rearing conditions and sampling*

The cod larvae were grown either in flow-through or recirculating tanks, and fed either intensively reared rotifers or mainly natural plankton (Table 1). Large and fit looking larvae were sampled from tanks with good larval growth and survival.

The intestinal bacterial flora was isolated by starving the sampled cod larvae for 3-4 hours to empty their guts, before 5-6 individual larvae from each rearing condition were anaesthetized (30 sec, metomidate 100 mg/l), surface disinfected (30 sec, 0.1% benzalkoniumcloride), and washed twice (2 x 60 sec) in sterile 80% seawater (Munro et al. , 1994). The larvae were then homogenized in 1 ml sterile 80% seawater, and serial dilutions of the homogenate was plated in duplicate on marine agar 2216 (Difco, USA). The agar plates were incubated for at least 12 days at 15°C.

*Screening for antagonistic isolates*

The screening for isolates with antagonistic activity against L. anguillarum HI 610 (kindly provided by Øyvind Bergh, Institute of Marine Research, Bergen, Norway) was performed with the replica-plating method (Hjelm et al. , 2004a). Agar plates with no

more than 100 colonies were replica-plated onto an agar plate containing 10 ml M9GC-3 agar. Prior to pouring the agar onto the plates, it was held at approximately 44°C and mixed with 10 µl per plate of one day old L. anguillarum HI 610 grown in marine broth 2216 (Difco, USA). After transferring the colonies using a sterile nitrocellulose membrane, the replica-plates were incubated for two days at 15°C, and then visually checked for clearing zones indicating antagonistic activity. Colonies with antagonistic activity were cultured to purity on marine agar 2216, regrown in marine broth 2216 for one day at 22°C and stored in glycerol (40% v/v) at -80°C. To assure that the assay was working properly, the two isolates Roseobacter sp. 27-4 (Hjelm et al. , 2004b) and Pseudomonas fluorescence AH2 (Gram et al. , 1999), both with strong antagonistic activity, were used as positive controls for each test.

*Phenotypic characterization and identification of bacterial isolates*

The isolates were phenotypically characterized on the basis of Gram reaction using both Bactident ® aminopeptidase strips (Merck 1.13301) and a 3% KOH solution (Gregersen, 1978), the ability to ferment and/or oxidize glucose in OF basal medium (Merck 1.10282) prepared with 80% seawater, oxidase reaction (Oxoid), motility and shape after growth for 1 day at 22°C in marine broth 2216 (phase contrast microscopy at 1000 times magnification), growth in marine broth 2216 with additional NaCl (final conc. 6%), sensitivity to O/129 (150 µg, Oxoid), and colony morphology (grown on marine agar 2216). The isolates were further characterized by some of the methods for phenotypical characterization described by Hansen and Sørheim (1991). We tested aerobic production of acid from fructose, mannose and glycerol, activity of urease, production of indole from tryptophan, methyl red test, and the ability to break down L-

arginine and L-lysine to alkaline amines under anaerobic conditions. The data was recorded in binary form and dendograms were prepared using average linkage and percent distance (Systat ver.9). The isolates were clustered at 80% similarity. The bacterial diversity in different hatcheries was calculated using the Shannon´s diversity index.

*Sequencing and phylogenetic analysis*

PCR template was produced by suspending colony material in 20 µl sterile water, incubate at 99°C for 10 min to lyse the bacterial cells, and centrifugation for 1 min to sediment cell constituents. DNA in the supernatant was amplified using 2 µl as template. The primers 8f (5`-AGA GTT TGA TC(AC) TGG CTC AG – 3`) and 517r (5`-ATT ACC GCG GCT GCT GG – 3`) were used in the PCR amplification to amplify ~500 bp of the 16S rDNA. The 25 µl reaction mixture included PCR-buffer (QIAGEN), dNTP mix (200 µM), $MgCl_2$ (0.5 mM), primers (0.4 µM each), 0.2 µl <u>Taq</u> DNA polymerase and template amplicon. PCR amplifications were performed using an initial denaturation at 95°C for 5 min followed by 25 cycles of denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min, and primer extension at 72°C for 1 min. The final extension was performed at 72°C for 8 min. Negative control was included in each PCR run. Agarose gel electrophoresis was run to verify PCR products and a 100 bp DNA ladder was included in each gel. Amplified DNA for sequencing was prepared by removing primers and nucleotides using a mix of 1 µl ExoSAP-IT (USB), 1 µl PCR product and 3.5 µl water. This mix was incubated at 37°C for 15 min followed by 80°C for 15 min. The reaction mix was then added 2 µl Big dye (Ver. 1.1, Applied Biosystems), 2 µl buffer (400 mM Tris-HCl pH 9, 10 mM $MgCl_2$) and sequencing

primer (8f, 0.7 µM) and incubated with an initial step at 96°C for 1 min followed by 25 cycles of 96°C for 1 min, 50°C for 15 sec, and 60°C for 4 min. The products from the sequencing reaction were precipitated by adding 2 µl Sodium Acetate (3 M, pH 5.2) and 100 µl EtOH to a 1.5 ml Eppendorf tube and put at -20°C for 20 min. The tubes were centrifuged at 13000 rpm for 30 min at 15°C. After centrifugation the supernatant was discarded, 250 µl EtOH was added and vortexed for 10 sec to wash the pellet. The mixture was centrifuged at 13000 rpm for 10 min, and the supernatant was carefully removed. The pellet was air dried at 50°C and stored at -20°C until sequencing was performed using ABI Prism 3100 Genetic Analyzer.

The DNA sequences were aligned to known sequences in the GenBank database using BLAST (Altschul et al. , 1990). Phylogenetic relationships were inferred using the neighbour joining method, based on the Kimura two-parameter model, in the PhylOgenetic WEb Repeater (Lin et al. , 2005). Reference strains that were obtained from the GenBank database were included in the alignment. We used the TreeView program to draw the phylogenetic tree. Sequences are deposited in the GenBank database under accession numbers **DQ 273862** to **DQ 273886**.

*Statistics*

ANOVA and Tukey´s multiple comparison test with arcsin-transformed data were used to compare the percentage of antagonistic isolates in larvae from different rearing groups.

**Results**

*Identification of the antagonistic isolates*

The cultivable intestinal bacterial flora of 27 individual cod larvae from the five rearing groups was screened for inhibitory activity against L. anguillarum (strain HI 610). Antagonistic bacteria were found within 18 of the 27 cod larvae, and 98 antagonistic strains were isolated. Based on phenotypical tests, the 98 antagonistic isolates were clustered (Figure 1), and at the 80% similarity level 12 bacterial clusters were identified (Table 2). The 25 isolates that were partially sequenced and aligned to sequences in the GenBank database represented 10 of the 12 bacterial clusters (Table 3). The majority of the DNA alignments showed relatedness to bacteria within the Gram negative γ-Proteobacteria (Marinomonas, Pseudoalteromonas, Shewanella and Vibrio), but two isolates had 16S rDNA similarity to isolates within α-Proteobacteria (Hyphomicrobium and Roseobacter) (Figure 2).

*Comparison within and between rearing groups*

The majority of antagonistic bacteria that were isolated from larvae reared in the two flow-through rearing groups (I1 and I2) belonged to cluster 12, causing a low diversity index (0.49 and 0.41, respectively) in these groups (Table 2). Bacteria in cluster 12 had high sequence similarities to Vibrio (Table 3). In the larvae fed natural plankton (N), the antagonistic bacteria in three larvae belonged to cluster 3, whereas the bacteria in the other two larvae belonged to cluster 12. The diversity index was higher in this group (0.69). In larvae that were reared using water recirculation technology (R1 and R2), the antagonistic bacteria were more numerous in one of the groups (R2) and the diversity

index was higher in both groups (1.21 and 1.50, respectively), compared to other the rearing regimes. The antagonistic isolates found in larvae from the two recirculation groups belonged to 10 of the 12 clusters (Table 1). Cluster 12 was represented in three larvae, and cluster 7 was represented in two larvae, whereas eight clusters (1, 2, 4, 6, 8, 9, 10 and 11) were represented only in one larva.

Antagonistic isolates belonging to cluster 12, with DNA similarity to Vibrio, were found in cod larvae from all five rearing conditions. The number of isolates that were grouped in this cluster was clearly the highest (32). On the other hand, bacterial isolates from nine clusters (1, 2, 3, 4, 6, 7, 8, 9 and 10) were only found in larvae from one of the rearing groups. They had DNA similarity to Hyphomicrobium, Marinomonas, Pseudoalteromonas, Roseobacter and Shewanella. In cod larvae from all five rearing groups, we found that one or several larvae (I1:2, I2:2, N:1, R1:3, R2:1) did not harbour detectable levels of cultivable isolates with antagonistic activity against L. anguillarum HI610.

The average percentage of antagonistic isolates (Figure 3) in individual cod larvae was significantly different in larvae from hatchery R2 (mean 41.6%), compared to larvae from the other hatcheries (range of means 1.0 - 5.1%, $P < 0.007$).

**Discussion**

The majority of the bacteria with antagonistic abilities that were isolated in this study were members of the γ-Proteobacteria. This was also found by Long and Azam (2001) when they investigated the antagonistic interactions among marine pelagic bacteria. Within the γ-Proteobacteria, isolates with 16S rDNA similarity to <u>Vibrio</u> were found in larvae from all five rearing groups and it was the most numerous antagonistic genus. <u>Vibrio</u> also constituted a major part of the antagonistic intestinal bacteria in Japanese flounder (Sugita et al. , 2002), and it was the only bacterial genus with inhibitory activity in the intestines of Senegalese sole (Makridis et al. , 2005). In cod larvae from one of the recirculation groups (R2), antagonistic isolates with 16S rDNA similarity to <u>Marinomonas</u> sp. were found in high numbers and within four different phenotypic clusters. In this rearing group high mortality of larvae was observed one week after our sampling, and <u>Marinomonas</u> was isolated in large numbers after this incidence. Thus, the antagonistic isolates with DNA similar to <u>Marinomonas</u>, could have been the cause of this mortality. <u>Pseudoalteromonas</u> and <u>Shewanella</u> were also found among the antagonistic intestinal bacterial flora in the cod larvae, but in small numbers. Several <u>Pseudoalteromonas</u> species have shown a broad range of inhibitory activities against bacteria and other organisms (Holmström et al. , 2002). The non γ-Proteobacteria antagonistic isolates that were isolated in this study were members of α-Proteobacteria, and the isolates had 16S sequence similarity to <u>Hyphomicrobium</u> and <u>Roseobacter</u>. The most numerous antagonistic bacteria in a turbot rearing system were <u>Roseobacter</u> spp (Hjelm et al. , 2004b), and the antagonistic <u>Roseobacter</u> sp. strain 27-4 showed probiotic potential by decreasing the mortality in yolk sac experiments with turbot larvae (Hjelm et al. , 2004a).

Both the composition and the percentage of antagonistic isolates in the intestinal bacterial flora varied considerably between individual cod larvae from the same rearing condition. Individual variation in the percent of intestinal bacteria with inhibitory activity was also found in turbot (Westerdahl et al. , 1991). Stochastic factors may be important in the colonization of larvae, and could explain these differences at the individual level (Verschuere et al. , 1997). Stochastic factors includes that chance favours those bacteria that happen to be in the right place at the right time to colonize the intestinal tract and proliferate. Inter-individual variation has also been found in the intestinal bacterial flora of broiler chickens (Zhu et al. , 2002), pigs (Simpson et al. , 2000) and human beings (Zoetendal et al. , 1998; Hopkins et al. , 2002).

Almost 60% of the antagonistic isolates were found in larvae from one of the hatcheries using water recirculation technology (R2). Moreover, the percentage of antagonistic bacteria in these larvae was significantly higher and the diversity index was higher compared to the other rearing conditions. The percentage of antagonistic isolates in this rearing group (average 41.6%) was comparable to the levels found in turbot (Westerdahl et al. , 1991) and Senegalese sole (Makridis et al. , 2005). Isolates with 16S sequence similarity to Marinomonas sp. was the dominating bacterial genera with antagonistic abilities in larvae from this rearing condition. The percentage of antagonistic isolates in the other four rearing groups (average 1.0 to 5.1%) was similar to what was found in turbot (Hjelm et al. , 2004b) and Japanese flounder (Sugita et al. , 2002). Within these four rearing groups, bacteria in clusters with sequence similarity to Vibrio were dominant. In contrast to the relatively low percentage (average 5.1%) of

antagonistic isolates we found in larvae fed natural plankton (hatchery N), the intestinal bacteria from Senegalese sole fed natural prey had a high percentage of isolates with antibacterial activity (Makridis et al. , 2005).

Most studies searching for probiotic candidates focus on bacteria that show inhibitory activity in an in vitro assay against a target pathogen (Gram and Ringø, 2005). The rationale behind this strategy is that when these probiotic bacteria are established in the gut, they will produce substances that inhibit growth and thus colonization by pathogenic bacteria. An alternative strategy is isolation of the dominant bacterial flora in well performing fish (Skjermo and Vadstein, 1999; Huys et al. , 2001). It is assumed that the dominant bacteria in the intestinal tract of healthy larvae are good candidates due to their high competitive ability in the gut habitat. In a parallel study (Fjellheim et al. in prep.) we isolated the dominant bacterial flora of cod larvae from three of the rearing groups in this study (I2, N and R2). By comparing the bacteria, and hence pools of probiotic candidates, obtained by using both approaches we found two major differences. 1) The diversity index for the dominant bacteria was higher than for the antagonistic bacteria. The Gram positive Microbacterium which dominated in two of the rearing groups, was not antagonistic against L. anguillarum HI610 and was thus not in the antagonistic pool of isolates. Other genera that were specific for the dominant pool were Halomonas, Neptunomonas and Pseudomonas. The two bacterial genera Hyphomicrobium and Shewanella were only found in the antagonistic pool of isolates, while isolates with DNA sequence similarities to Marinomonas, Pseudoalteromonas, Roseobacter and Vibrio were found in both groups. 2) The total number of bacterial isolates that were obtained was different between the two selection strategies. Following

the dominant strategy, we isolated 30 bacterial colonies from each cod larva, whereas we isolated on average less than 4 antagonistic bacterial colonies from each larvae.

This study and previous studies (Long and Azam, 2001; Sugita et al. , 2002; Grossart et al. , 2004) have shown that a range of marine bacterial genera produce antimicrobial compounds in in vitro tests. In vitro test conditions are standardized with regards to growth media and temperature, and they usually only test two bacterial isolates against each other. The in vivo habitat is far more complex. The growth may be restricted due to availability of food, temperatures may change and many bacterial species are usually present at the same time. It has been shown that antagonism is differently expressed when tested on different growth media (Bizani and Brandelli, 2004; Hjelm et al. , 2004a). As the available food in the gut of marine fish larvae will change over time, it is difficult to predict if the bacteria will produce antimicrobial compounds in the in vivo situation. Also, antagonistic compounds are often only produced during the stationary growth phase in in vitro tests (Bizani and Brandelli, 2004; Monteiro et al. , 2005), which raises the question whether the bacteria will ever produce antagonistic compounds in the gut of fish larvae. Even though several conditions throw suspicion on the production of antimicrobial compounds in vivo by bacteria, it is hard to imagine how this common bacterial quality should have evolved and persisted if it did not have ecological implications in vivo.

**Acknowledgments**

## References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J. (1990) Basic local alignment search tool. J. Mol. Biol. 215:403-410.

Atlas, R.M. (1999) Probiotics - snake oil for the new millennium? Environ. Microbiol. 1:377-382.

Bizani, D., Brandelli, A. (2004) Influence of media and temperature on bacteriocin production by Bacillus cereus 8A during batch cultivation. Appl. Microbiol. Biotechnol. 65:158-162.

Fjellheim, A.J., Playfoot, K.J., Skjermo, J., Vadstein, O. Large inter-individual variation in the dominant intestinal bacterial flora of reared cod larvae. In prep.

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

Gram, L., Ringø, E. (2005) Prospects of fish probiotics. In: Holzapfel, W., Naughton, P. (Eds.) Microbial ecology of growing animals. Elsevier.

Gregersen, T. (1978) Rapid method for distinction of gram-negative from gram-positive bacteria. Eur. J. Appl. Microbiol. Biotechnol. 5:123-127.

Grossart, H.P., Schlingloff, A., Bernhard, M., Simon, M., Brinkhoff, T. (2004) Antagonistic activity of bacteria isolated from organic aggregates of the German Wadden Sea. FEMS Microbiol. Ecol. 47:387-396.

Hansen, G.H., Sørheim, R. (1991) Improved method for phenotypical characterization of marine bacteria. J. Microbiol. Methods 13:231-241.

Hentges, D.J. (1992) Gut flora and disease resistance. In: Fuller, R. (Ed.) Probiotics. The scientific basis. Chapman & Hall, London, pp 87-110.

Hjelm, M., Bergh, Ø., Riaza, A., Nielsen, J., Melchiorsen, J., Jensen, S., Duncan, H., Ahrens, P., Birkbeck, H., Gram, L. (2004a) Selection and identification of autochthonous potential probiotic bacteria from turbot larvae (Scophthalmus maximus) rearing units. Syst. Appl. Microbiol. 27:360-371.

Hjelm, M., Riaza, A., Formoso, F., Melchiorsen, J., Gram, L. (2004b) Seasonal incidence of autochthonous antagonistic Roseobacter spp. and Vibrionaceae strains in a turbot larva (Scophthalmus maximus) rearing system. Appl. Environ. Microbiol. 70:7288-7294.

Holmström, C., Egan, S., Franks, A., McCloy, S., Kjelleberg, S. (2002) Antifouling activities expressed by marine surface associated Pseudoalteromonas species. FEMS Microbiol. Ecol. 41:47-58.

Hopkins, M.J., Sharp, R., Macfarlane, G.T. (2002) Variation in human intestinal microbiota with age. Dig. Liver Dis. 34:S12-S18.

Huys, L., Dhert, P., Robles, R., Ollevier, F., Sorgeloos, P., Swings, J. (2001) Search for beneficial bacterial strains for turbot (Scophthalmus maximus L.) larviculture. Aquaculture 193:25-37.

Lin, C.Y., Lin, F.K., Lin, C.H., Lai, L.W., Hsu, H.J., Chen, S.H., Hsiung, C.A. (2005) POWER: PhylOgenetic WEb Repeater - integrated and user-optimized framework for biomolecular phylogenetic analysis. Nucleic Acids Res. 33:W553-W556.

Long, R.A., Azam, F. (2001) Antagonistic interactions among marine pelagic bacteria. Appl. Environ. Microbiol. 67:4975-4983.

Makridis, P., Martins, S., Tsalavouta, M., Dionisio, L.C., Kotoulas, G., Magoulas, A., Dinis, M.T. (2005) Antimicrobial activity in bacteria isolated from Senegalese sole, Solea senegalensis, fed with natural prey. Aquacult. Res. 36:1619-1627.

Monteiro, L., Mariano, R.D.R., Souto-Maior, A.M. (2005) Antagonism of Bacillus spp. against Xanthomonas campestris pv. campestris. Braz. Arch. Biol. Technol. 48:23-29.

Munro, P.D., Barbour, A., Birkbeck, T.H. (1994) Comparison of the gut bacterial-flora of start-feeding larval turbot reared under different conditions. J. Appl. Bacteriol. 77:560-566.

Olafsen, J.A. (2001) Interactions between fish larvae and bacteria in marine aquaculture. Aquaculture 200:223-247.

Ringø, E., Gatesoupe, F.J. (1998) Lactic acid bacteria in fish: a review. Aquaculture 160:177-203.

Simpson, J.M., McCracken, V.J., Gaskins, H.R., Mackie, R.I. (2000) Denaturing gradient gel electrophoresis analysis of 16S ribosomal DNA amplicons to monitor changes in fecal bacterial populations of weaning pigs after introduction of Lactobacillus reuteri strain MM53. Appl. Environ. Microbiol. 66:4705-4714.

Skjermo, J., Salvesen, I., Øie, G., Olsen, Y., Vadstein, O. (1997) Microbially matured water: A technique for selection of a non-opportunistic bacterial flora in water that may improve performance of marine larvae. Aquacul. Int. 5:13-28.

Skjermo, J., Vadstein, O. (1999) Techniques for microbial control in the intensive rearing of marine larvae. Aquaculture 177:333-343.

Sugita, H., Okano, R., Suzuki, Y., Iwai, S., Mizukami, M., Akiyama, N., Matsuura, S. (2002) Antibacterial abilities of intestinal bacteria from larval and juvenile Japanese flounder against fish pathogens. Fish. Sci. 68:1004-1011.

Sugita, H., Shibuya, K., Shimooka, H., Deguchi, Y. (1996) Antibacterial abilities of intestinal bacteria in freshwater cultured fish. Aquaculture 145:195-203.

Vadstein, O., Mo, T.A., Bergh, Ø. (2004) Microbial interactions, prophylaxis and diseases. In: Moksness E, Kjørsvik E, Olsen Y (Eds.) Culture of cold-water marine fish. Blackwell Publishing Ltd, pp 28-72.

Verschuere, L., Dhont, J., Sorgeloos, P., Verstraete, W. (1997) Monitoring Biolog patterns and r/K-strategists in the intensive culture of Artemia juveniles. J. Appl. Microbiol. 83:603-612.

Westerdahl, A., Olsson, J.C., Kjelleberg, S., Conway, P.L. (1991) Isolation and characterization of turbot (Scophthalmus maximus)-associated bacteria with inhibitory effects against Vibrio anguillarum. Appl. Environ. Microbiol. 57:2223-2228.

Zhu, X.Y., Zhong, T.Y., Pandya, Y., Joerger, R.D. (2002) 16S rRNA-based analysis of microbiota from the cecum of broiler chickens. Appl. Environ. Microbiol. 68:124-137.

Zoetendal, E.G., Akkermans, A.D.L., De Vos, W.M. (1998) Temperature gradient gel electrophoresis analysis of 16S rRNA from human fecal samples reveals stable and host-specific communities of active bacteria. Appl. Environ. Microbiol. 64:3854-3859.

Table 1. Rearing conditions of the cod larvae that were sampled from five Norwegian cod hatcheries.

| Hatchery | Hatchery ID | Rearing technology | Feed | Sampling time (day degrees) |
|---|---|---|---|---|
| Fosen akvasenter | I1 | Flow-through water | Intensively reared rotifers | 99 |
| Brattøra Research Center | I2 | Flow-through water | Intensively reared rotifers | 190 |
| Lofilab | N | Flow-through water | Mainly natural zooplankton | 179 |
| Fosen akvasenter | R1 | Recirculating water | Intensively reared rotifers | 190 |
| Troms Marin Yngel | R2 | Recirculating water | Intensively reared rotifers | 216 |

Table 2. Number of antagonistic isolates belonging to each cluster in individual larvae, and diversity index from the five rearing groups. No antagonistic isolates were found in 9 of the 27 larvae that were screened, these larvae are not shown.

| Cluster no | Flow through water | | | | | | | | Recirculated water | | | | | | | | | Fed natural plankton | | | | | | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | IA 6 | IA 7 | IA 8 | IA 9 | Sum 11 | IA 4 | IA 5 | Sum 12 | RA 2 | RA 3 | Sum R 1 | RA 4 | RA 5 | RA 6 | RA 7 | RA 9 | Sum R 2 | N A1 | N A2 | N A3 | N A4 | N A6 | Sum NA | |
| 1 | | | | | 0 | | | 0 | | 1 | 1 | | | | | | 0 | | | | | | 0 | 1 |
| 2 | | | | | 0 | | | 0 | | | 0 | | 3 | | | | 3 | | | | | | 0 | 3 |
| 3 | | | | | 0 | | | 0 | | | 0 | | | | | | 0 | 3 | | | 1 | 1 | 5 | 5 |
| 4 | | | | | 0 | | | 0 | | 1 | 1 | | | | | | 0 | | | | | | 0 | 1 |
| 5 | | | | 1 | 1 | | 1 | 1 | | | 0 | | | | | | 0 | | | | | | 0 | 2 |
| 6 | | | | | 0 | | | 0 | | | 0 | | | 16 | | | 16 | | | | | | 0 | 16 |
| 7 | | | | | 0 | | | 0 | | | 0 | 7 | | | 18 | | 25 | | | | | | 0 | 25 |
| 8 | | | | | 0 | | | 0 | | | 0 | | | | | 6 | 6 | | | | | | 0 | 6 |
| 9 | | | | | 0 | | | 0 | | | 0 | | 1 | | | | 1 | | | | | | 0 | 1 |
| 10 | | | | | 0 | | | 0 | | | 0 | | 2 | | | | 2 | | | | | | 0 | 2 |
| 11 | | | | 1 | 1 | | | 0 | | 3 | 3 | | | | | | 0 | | | | | | 0 | 4 |
| 12 | 4 | 3 | 4 | 2 | 13 | 4 | 2 | 6 | 3 | 1 | 4 | | 5 | | | | 5 | | 3 | 1 | | | 4 | 32 |
| Sum | 4 | 3 | 4 | 4 | 15 | 4 | 3 | 7 | 3 | 6 | 9 | 7 | 11 | 16 | 18 | 6 | 58 | 3 | 3 | 1 | 1 | 1 | 9 | 98 |
| Diversity index | | | | | 0.49 | | | 0.41 | | | 1.21 | | | | | | 1.50 | | | | | | 0.69 | |

Table 3. Relationship between sequenced isolates and other sequences in GenBank.

| Cluster | Isolate | Accession number | Closest match | Accession number | Similarity (%) | Taxon |
|---------|---------|------------------|---------------|------------------|----------------|-------|
| 2 | RA 5-2 | DQ273873 | Pseudoalteromonas sp. 3041 | AM110994 | 98 | γ-Proteobacteria |
| 2 | RA 5-5 | DQ273879 | Pseudoalteromonas sp. 7026 | AM111027 | 95 | γ-Proteobacteria |
| 4 | RA 3-4 | DQ273866 | Shewanella aquimarina | AY485225 | 93 | γ-Proteobacteria |
| 5 | IA 5-3 | DQ273886 | Marine gamma proteobacterium LHFL1 | AF366029 | 80 | γ-Proteobacteria |
| 6 | RA 6-2 | DQ273870 | Marinomonas sp. CK16 | AM084271 | 99 | γ-Proteobacteria |
| 6 | RA 6-4 | DQ273872 | Marinomonas sp. CK16 | AM084271 | 99 | γ-Proteobacteria |
| 6 | RA 6-7 | DQ273874 | Marinomonas sp. CK16 | AM084271 | 98 | γ-Proteobacteria |
| 6 | RA6-12 | DQ273877 | Marinomonas sp. CK16 | AM084271 | 99 | γ-Proteobacteria |
| 7 | RA 4-1 | DQ273862 | Roseobacter sp. JL-126 | AY745859 | 98 | α-Proteobacteria |
| 7 | RA 7-14 | DQ273863 | Pseudoalteromonas sp. 8050 | AM111084 | 99 | γ-Proteobacteria |
| 7 | RA 7-11 | DQ273884 | Marinomonas sp. CK16 | AM084271 | 99 | γ-Proteobacteria |
| 8 | RA 9-5 | DQ273864 | Marinomonas sp. CK16 | AM084271 | 97 | γ-Proteobacteria |
| 8 | RA 9-6 | DQ273868 | Marinomonas sp. CK16 | AM084271 | 92 | γ-Proteobacteria |
| 8 | RA 9-2 | DQ273882 | Marinomonas sp. CK16 | AM084271 | 97 | γ-Proteobacteria |
| 9 | RA 5-11 | DQ273867 | Marinomonas sp. CK16 | AM084271 | 90 | γ-Proteobacteria |
| 10 | RA 5-8 | DQ273881 | Hyphomicrobium sp. YAAJ-4 | DQ200849 | 95 | α-Proteobacteria |
| 11 | RA 3-6 | DQ273869 | Vibrio ordalii strain 2003/09/511-2063 | AY530930 | 98 | γ-Proteobacteria |
| 11 | RA 3-1 | DQ273883 | Uncultured bacterium clone ISA-7084 | AY911193 | 92 | Unknown |
| 12 | IA 4-3 | DQ273865 | Vibrio splendidus strain LP1 | AY129277 | 98 | γ-Proteobacteria |
| 12 | IA 5-2 | DQ273871 | Vibrio ordalii NCMB2168 | AY628646 | 99 | γ-Proteobacteria |
| 12 | IA 8-2 | DQ273875 | Vibrio sp. 3d clone 3d4 | AF388392 | 98 | γ-Proteobacteria |
| 12 | RA 5-4 | DQ273876 | Uncultured Vibrio sp. clone SIMO-713 | AY712250 | 97 | γ-Proteobacteria |
| 12 | NA 2-2 | DQ273878 | Vibrio sp. V051 | DQ146974 | 98 | γ-Proteobacteria |
| 12 | RA 2-3 | DQ273880 | Vibrio aestuarianus | AJ845021 | 99 | γ-Proteobacteria |
| 12 | IA 5-1 | DQ273885 | Marine gamma proteobacterium LHFL1 | AF366029 | 83 | γ-Proteobacteria |

Figure legends:

Figure 1. Cluster diagram for antagonistic isolates using average linkage and percent distance. The isolates were clustered based on phenotypic tests at 80% similarity, as indicated by the dotted line.

Figure 2: Phylogenetic tree showing the positions of bacterial 16S rDNA sequences recovered from the five different rearing conditions. Scale bar indicates 10% sequence difference. The 16S rDNA sequence of <u>Bacillus cereus</u> (**AF155952**) was used as outgroup reference. Accession numbers of reference sequences are indicated. The number after the asterisk refers to the cluster where isolates were placed according to the biochemical tests.

Figure 3. Percentage antagonistic isolates of total colony forming units in individual larvae from the five different rearing conditions. Cicles (○) indicate individual larvae, the boxes show $25^{th}$ and $75^{th}$ percentiles and median. The dotted lines are the mean values.
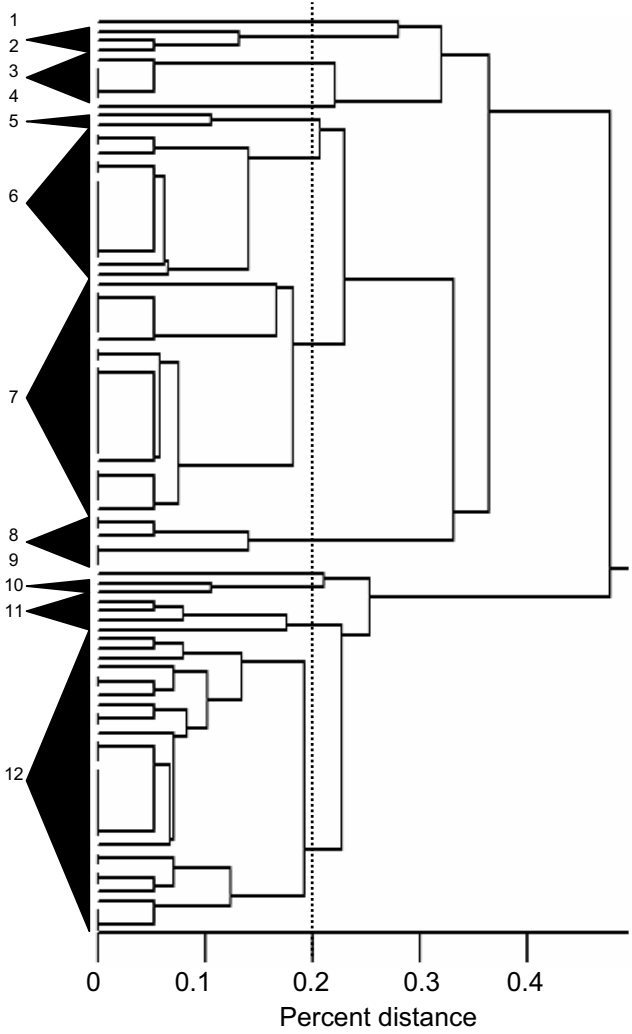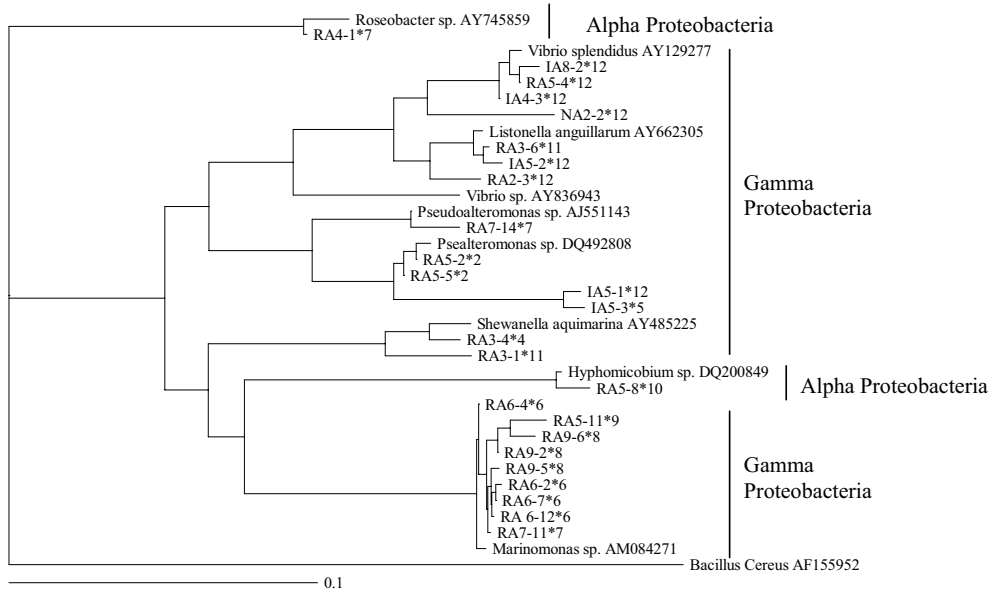
**Figure 1**

**Figure 2**

**Figure 3**
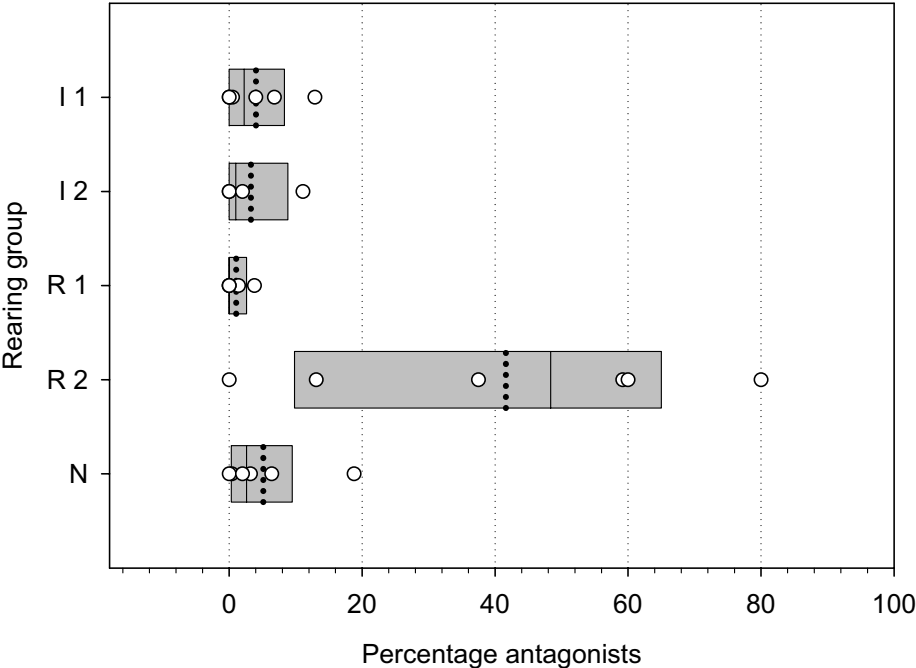
# Paper III

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# Paper IV

# Control of the bacterial flora of *Brachionus plicatilis* and *Artemia franciscana* by incubation in bacterial suspensions

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## Abstract

The accumulation of bacteria in *Brachionus plicatilis* and *Artemia franciscana* during a short-term incubation was quantified using immunocolony blot (ICB) and an enzyme-linked immunosorbent assay (ELISA). Four bacterial strains, isolated from turbot and halibut, were grazed effectively by both species when given at high concentrations ($\geq 5 \times 10^7$ bacteria ml$^{-1}$). *B. plicatilis* accumulated $21$–$63 \times 10^3$ bacteria per rotifer and *A. franciscana* up to $45 \times 10^3$ bacteria per metanauplius after 20–60 min of grazing. The composition of the bacterial microflora of the live food organisms changed drastically, as the bioencapsulated strains comprised up to 100% of the total count of colony-forming units. After incubation in the bacterial suspensions, *B. plicatilis* and *A. franciscana* were transferred to seawater with added microalgae (*Tetraselmis* sp., 2 mg C l$^{-1}$), to evaluate the persistence of the changed bacterial composition in conditions similar to those present in a first feeding tank. The bioencapsulated bacteria decreased in numbers, but in most cases remained present in both live food organisms after 24 h. It is possible, after a short-term incubation, to replace opportunistic (r-selected) bacteria present in the live food cultures with other bacteria, which persist as a dominant part of the bacterial flora of the live food for a relatively long period of time (4–24 h). © 2000 Elsevier Science B.V. All rights reserved.

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## 1. Introduction

Intensive rearing of marine fish larvae suffers from heavy mortalities, which may be attributed to bacteria introduced in the rearing system with live food (Nicolas et al., 1989; Keskin et al., 1994). The high organic load associated with intensive production of live food cultures selectively induces an increased proportion of opportunistic (r-selected) bacteria, which may be pathogenic to the fish larvae (Skjermo and Vadstein, 1999). Disinfection, although beneficial, may not prevent a re-colonization of the live food within a short time period (Munro et al., 1999). Replacement of the opportunistic bacteria with other less-aggressive bacteria may provide a solution.

The rotifer *Brachionus plicatilis* and the brine shrimp *Artemia franciscana* are common live food organisms used for the rearing of marine fish larvae. These have been considered as possible vectors for the delivery of different substances, such as nutrients (Watanabe et al., 1983), antimicrobial agents (Mohney et al., 1990; Dixon et al., 1995), vaccines (Campbell et al., 1993), and probiotics (Gatesoupe, 1994). These are live bacterial additives that may have a positive effect on the host organism by improving the properties of the indigenous microflora (Havennar et al., 1992). This positive effect of probiotics may be attributed to their ability to outcompete other bacteria (Austin et al., 1995), or to produce micronutrients important for the development of fish larvae (Sugita et al., 1991; Ringø et al., 1992).

The quantitative and qualitative properties of the bacterial microflora of live food must be adjusted to avoid the negative effects of an overload of bacteria (Benavente and Gatesoupe, 1988; Nicolas et al., 1989; Skjermo and Vadstein, 1993; Keskin et al., 1994), and at the same time accomplish a successful colonization of the larval gut (Munro et al., 1999). A short-term incubation of live food organisms in a bacterial suspension consisting of one or several probiotic strains is a possible approach to replace opportunists with other less-aggressive bacteria. Live food organisms may reside in the rearing tanks for several hours before ingested by the larvae (Reitan et al., 1993). Once the bacteria have been bioencapsulated in the live food, it is important to determine the rate of loss of the bioencapsulated bacteria, and whether the changed bacterial composition persists, as live food organisms may be depleted of the specific bacteria before they are ingested by fish larvae. The detection of specific bacteria in the live food or the larvae, has been accomplished by use of enzyme-linked immunosorbent assay (ELISA) (Ringø et al., 1996), measuring the amount of lipopolysaccharide (Kawai et al., 1989), or by counting the number of colony-forming units (CFU) (Gomez-Gil et al., 1998).

The aims of this study were: (i) to control the composition of the bacterial flora of live food by adding specific bacteria to the culture water and letting them feed for a short time period, (ii) to evaluate if the changed bacterial composition was maintained when the live food organisms were transferred to conditions similar to those in a first feeding tank, and (iii) to compare two types of immunoassays as methods for detecting specific bacteria in the live food. For comparative purposes, the live food organisms *B. plicatilis* and *A. franciscana* were used, and two bacterial strains were tested for each of the live food organisms.

## 2. Materials and methods

### 2.1. Bacteria

In the experiments with rotifers (*B. plicatilis*), we used two bacterial strains isolated from the gut of turbot (*Scophthalmus maximus* L.). The bacterial strain 4:44 was isolated from adult turbot and supplied by Ana Jöborn, University of Göteborg, Sweden (Olsson et al., 1992), whereas the bacterial strain PB52 (an unidentified Gram negative, motile, facultative anaerobe) was isolated at the SINTEF Center of Aquaculture from the gut flora of 15-day-old turbot larvae fed successively with *B. plicatilis* and *A. franciscana*.

In the experiments with *A. franciscana*, we used two *Vibrio* spp. strains, PB111 and PB61, which were supplied by Øivind Bergh, Institute of Marine Research, Norway. These bacteria were isolated from the gut microflora of halibut larvae (*Hippoglossus hippoglossus* L.) fed copepods (Bergh, 1995). All bacterial strains used, except PB52, have shown in vitro inhibition of pathogenic *Vibrio* strains (Olsson et al., 1992; Bergh, 1995). The bacterial strains used were not chosen for their probiotic activity, but were merely used as model organisms.

Bacteria were cultured at room temperature (20–22°C) in marine broth 2216 (Difco, USA) under continuous agitation. The total number of cells was determined after filtration onto black polycarbonate filters (0.2-$\mu$m pore size) by pre-staining with either DAPI or acridine orange (Hobbie et al., 1977; Porter and Feig, 1981), and observation in a Nikon Optiphot epifluorescence microscope. Upon harvest, the growth medium was removed after centrifugation at 5200–5500 rpm, and the cells were re-suspended in autoclaved seawater.

### 2.2. Rotifer experiments

*B. plicatilis* (SINTEF-strain, 250-$\mu$m average maximum length) were cultured in 200-l cultures in cylindroconical tanks, and fed bakers' yeast (*Saccharomyces cerivisiae*) and DHA-Selco (INVE Aquaculture NV, Belgium) at a 10:1 weight ratio (Olsen et al., 1993). The rotifers were harvested, rinsed with seawater, and starved for 2 h before the onset of the experiments. The rotifers were incubated at a density of approximately 200 rotifers $ml^{-1}$ in one-litre suspensions of bacteria for 60 min at 20–22°C under continuous aeration. Samples from the bacterial suspensions were plated on marine agar 2216 (Difco) before the onset of the experiments, to determine the number of CFU $ml^{-1}$. Samples of rotifers were taken before transfer to the bacterial suspensions and 0, 20, 40 and 60 min after transfer. At each sampling, 5.0 ml of rotifer culture were sieved on a 50 $\mu$m mesh, rinsed with 50 ml autoclaved seawater, and homogenized under sterile conditions. Serial dilutions of the homogenate were prepared in autoclaved 80% seawater, plated in duplicate on marine agar 2216 (Difco), and incubated for 5–7 days at 15°C. Samples of rotifer homogenate for detection of bacteria by ELISA were stored at −20°C.

After incubation in the bacterial suspensions, part of the rotifers were transferred for 24 h (20–22°C) to one-litre beakers with autoclaved seawater to which *Tetraselmis* sp. had been added (2 mg C $l^{-1}$), to simulate the conditions in a fish tank during first

feeding of turbot larvae. Population density was adjusted to 5 rotifers $ml^{-1}$, and samples of rotifers were taken, as previously described, 2, 4 and 24 h after transfer.

In the first experiment, rotifers were incubated in suspensions ($n = 3$) of the strain 4:44 ($1.2 \times 10^7$ CFU $ml^{-1}$). In the second experiment, rotifers were incubated in suspensions ($n = 2$) of the strain 4:44 at a higher concentration ($2.1 \times 10^8$ CFU $ml^{-1}$). A part of the rotifers were rinsed and transferred after 40 min of incubation in the bacterial suspensions to seawater with added algae. In the third experiment, suspensions ($n = 2$) of the strain PB52 ($3.3 \times 10^8$ CFU $ml^{-1}$) were used, and a part of the rotifers were rinsed and transferred to seawater with added algae after 20 min of incubation in the bacterial suspensions. In the fourth experiment, rotifers were incubated in a mixture ($n = 2$) of equal numbers of the two strains (total density of $3.5 \times 10^8$ CFU $ml^{-1}$), where a part of the rotifers was transferred to seawater with added algae after a 20 min incubation in the bacterial suspensions.

## 2.3. Artemia experiments

*A. franciscana* (EG-grade, INVE Aquaculture NV, Belgium) cysts were decapsulated, incubated for 24 h at 28°C and 5‰ salinity under strong illumination and aeration, and fed for 3 days after hatching with *Tetraselmis* sp. at 28°C. Three-day-old *A. franciscana* metanauplii were rinsed and transferred to one-litre suspensions of the bacteria for 60 min at 20–22°C. Three different concentrations in the range of $10^7–10^8$ bacteria $ml^{-1}$ were tested for each of the two bacterial strains, and the effect of *A. franciscana* density (25 and 50 *A. franciscana* $ml^{-1}$) was examined at each of these concentrations. Samples from the bacterial suspensions were taken at the onset of the experiments and after 30 min of grazing, diluted in 80% autoclaved seawater and plated on marine agar to determine the CFU $ml^{-1}$.

A part of *A. franciscana* that had grazed in the bacterial suspensions for 60 min was incubated for 24 h at 10–12°C, at a density of 1–2 *A. franciscana* $ml^{-1}$ in autoclaved seawater with added *Tetraselmis* sp. (2 mg C $l^{-1}$), to simulate first feeding conditions of halibut larvae. Samples of *A. franciscana* were taken, before the transfer to the bacterial suspensions, after 0, 30 and 60 min of incubation in the bacterial suspensions, and 1, 2 and 24 h after transfer to seawater with added algae.

During sampling, 10 *A. franciscana* metanauplii were rinsed with autoclaved seawater and homogenized in 5.0 ml autoclaved seawater. Serial dilutions of the homogenate in 80% seawater were plated on marine agar, and incubated at 15°C for 5–7 days. Samples of homogenate for the detection of bacteria by ELISA were stored at $-20$°C.

## 2.4. Antisera

Rabbit polyclonal antisera were prepared against the bacterial strains after immunization with whole cells, which had been fixed (0.5% formaldehyde), and washed twice in PBS. The antiserum against strain 4:44 showed no cross-reactions with strain PB52 and vice versa. Cross-reactions of the antisera against the other two bacterial strains (PB111

and PB61) were removed by incubating the antisera in a PBS solution with formalin-fixed whole cells of the cross-reacting bacteria for 3 h. The bacteria and the cross-reacting fraction of the antibodies were removed from the solution by centrifugation at 5000–5500 rpm for 15 min. The primary antisera were purified by use of a protein A purification column (Ey et al., 1978). A part of the purified antisera was biotinylated with NHS-LC-biotin (Pierce, USA).

### 2.5. ICB

ICB was used to detect colonies of the specific bacteria samples in seawater, and in *B. plicatilis* and *A. franciscana* homogenates (Kotani and McGarrity, 1986). Nitrocellulose membranes were placed on marine agar plates, and impressions of the colonies grown on the agar surface were made. The nitrocellulose membranes were successively incubated in primary polyclonal antibody (1:3000–4000), secondary goat anti-rabbit antiserum (1:2000) conjugated to horseradish peroxidase (Biorad, USA), and in HRP colour development reagent (0.05% w/v 4-chloro 1-naphthol, 0.16% v/v ethanol in ice-cold Tris-base saline).

### 2.6. ELISA

A sandwich-ELISA was used (Adams, 1991), where polystyrene Nunc Maxisorb plates were sensitized against the bacteria to be detected with purified rabbit antiserum (10 $\mu$g ml$^{-1}$ in 0.05 M bicarbonate buffer, pH 8.5). We added 100 $\mu$l of each sample in two-fold dilutions in PBS and incubated for 2 h. Biotinylated rabbit antiserum (1:1000) in PBS was applied for 1 h, and thereafter neutravidine conjugated with horse radish peroxidase was added (1:1000) for 30 min. *o*-Phenylenediamine (0.4 mg ml$^{-1}$) was used as a substrate in a phosphate–citrate buffer (0.5 M) added urea hydrogen peroxide (0.4 mg ml$^{-1}$). The reaction was stopped with 100 $\mu$l of 3 M HCl, and was quantified by measuring OD$_{492}$. Three washings (PBS with 0.5% v/v Tween-20) were applied between each incubation step of the assay (20–22°C).

Standard curves at OD$_{492}$ were established for the four bacterial strains. Bacterial densities were determined by plating diluted samples on marine agar, and by counting the total number of cells by epifluorescent microscopy. In the standards used, the numbers obtained by epifluorescent microscopy and colony counts were approximately equal.

## 3. Results

### 3.1. Rotifers

No positive reaction was shown between plated homogenate of control rotifers and antiserum against strain 4:44 in the first two experiments. In the third and fourth

experiments, the percentage of colonies from control rotifer homogenate reacting with the antisera against the strains 4:44 and PB52 was 17% and 15%, respectively. Total count of CFUs in rotifers that grazed suspensions of strain 4:44 at low concentrations $(1.2 \pm 0.5 \times 10^7 \text{ CFU ml}^{-1})$ was $1.1 \pm 0.3 \times 10^3$ CFU per rotifer after 20–60 min (Fig. 1), where 28–52% of the colonies were identified as 4:44 by ICB (Table 1). The numbers of strain 4:44 accumulated after 20, 40 and 60 min of grazing $(0.5 \pm 0.2 \times 10^3$ bacteria per rotifer) were not significantly different from the number measured at the 0 min sample $(P = 0.17, 0.08, 0.07,$ respectively), but strain 4:44 as a percentage of total CFU was significantly higher after 40 min than after 0 min $(P < 0.03)$.

Grazing of bacteria was more efficient at higher bacterial concentrations $(1.8–3.5 \times 10^8 \text{ CFU ml}^{-1})$, where $32–71 \times 10^3$ CFU of the bacterial strains per rotifer were accumulated within 20–60 min (Fig. 1). The number accumulated was significantly higher than in the 0-min sample $(P < 0.02)$. The bacterial strains, as a percentage of the total CFU in the rotifers, increased to 94–98% after the short-term enrichment (Table 1). Strain 4:44 was more effectively accumulated in the rotifers when given together with strain PB52, whereas strain PB52 was more effectively accumulated when given alone. In three out of four experiments with concentrated bacterial suspensions, the number of the specific bacteria per rotifer was lower after 60 min of enrichment than after 20 min of enrichment, when analysed by ICB. This tendency was not observed when the same samples were analysed by ELISA (Fig. 2). In general, the values obtained by ELISA during the enrichment phase, were higher than the values obtained by ICB. After transfer to seawater with added algae, the values obtained by the two methods were similar. The detection limit of bacteria using ELISA was $2–5 \times 10^3$ bacteria ml$^{-1}$.

After transfer to seawater with added algae, the rotifers lost most of their bacterial content (Figs. 1 and 2). The total CFU decreased on average for all experiments to 10.5, 4.6, and $3.4 \times 10^3$ CFU per rotifer after 2, 4, and 24 h, respectively. The percentage of



Fig. 1. Content of the bacterial strains 4:44 and PB52 (avg ± SEM) in rotifers as measured by ICB after 20, 40, and 60 min in suspensions of bacteria, and after transfer to seawater with added algae for 2, 4 and 24 h, indicated as 2A, 4A and 24A, respectively. The concentrations of bacteria were: ▲ $7.8 \times 10^6$ 4:44 ml$^{-1}$, ● $1.7 \times 10^8$ 4:44 ml$^{-1}$, □ mixture of equal numbers of 4:44 and PB52 (totally $3.5 \times 10^8$ bacteria ml$^{-1}$), and ◆ $3.3 \times 10^8$ PB52 ml$^{-1}$.

Table 1
The bacterial strains 4:44 and PB52 as a percentage of the total CFU (avg $\pm$ SEM) in *B. plicatilis* after 20, 40 and 60 min of grazing in the bacterial suspensions, and after transfer to seawater with added algae for 2, 4 and 24 h, indicated as 2A, 4A, 24A, respectively. The average concentration of the specific bacteria in the bacterial suspensions (CFU ml$^{-1}$) is also given

| Concentration | 4:44 | | PB52 | Mixture | |
|---|---|---|---|---|---|
| | | | | 4:44 | PB52 |
| | $1.7 \times 10^7$ | $1.8 \times 10^8$ | $3.3 \times 10^8$ | $1.7 \times 10^8$ | $1.7 \times 10^8$ |
| 20 min | $28 \pm 5$ | $98 \pm 1$ | $87 \pm 4$ | $62 \pm 1$ | $50 \pm 7$ |
| 40 min | $52 \pm 7$ | $94 \pm 1$ | | | |
| 60 min | $52 \pm 22$ | $90 \pm 6$ | $88 \pm 7$ | $44 \pm 7$ | $42 \pm 8$ |
| 2A | | $60 \pm 15$ | $60 \pm 16$ | $43 \pm 24$ | $66 \pm 5$ |
| 4A | | $65 \pm 5$ | $44 \pm 4$ | $26 \pm 14$ | $41 \pm 2$ |
| 24A | | $36 \pm 19$ | $16 \pm 1$ | $13 \pm 4$ | $26 \pm 8$ |

the specific bacteria, after 2, 4, and 24 h in seawater with added algae, decreased on average to 72%, 60% and 32%, respectively. Even after the rotifers had grazed for 24 h in a suspension of algae, the composition of their bacterial flora was different from the one in non-treated rotifers, where colonies giving a positive reaction in the ICB assay was $\leq$ 17% of the total CFU.

## 3.2. Artemia experiments

No positive reaction was observed between plated homogenate of control metanauplii and the antisera against the strains PB111 and PB61. The population density of
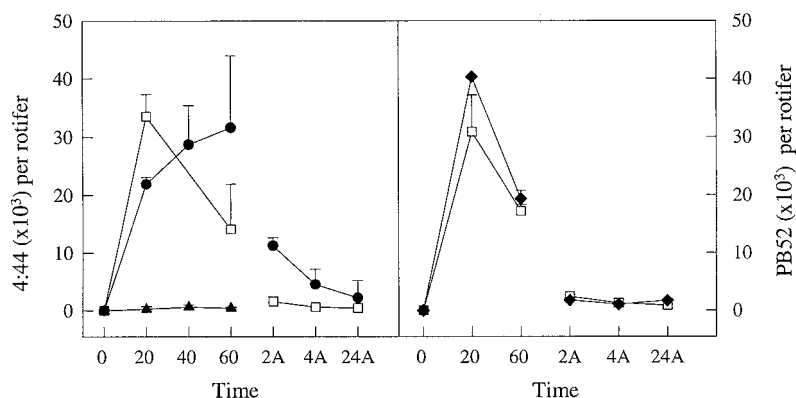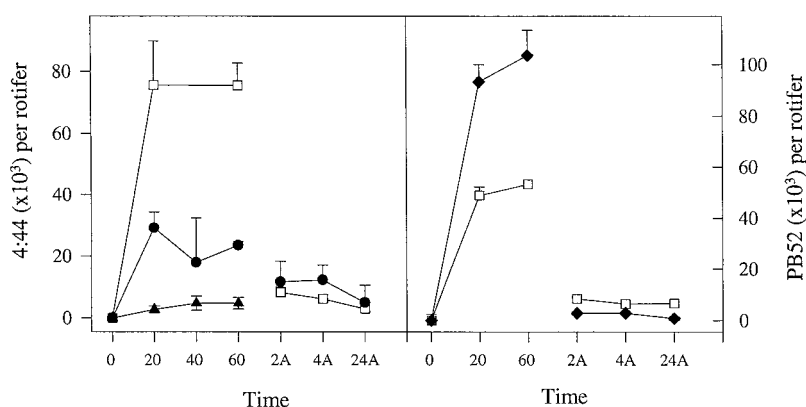


Fig. 2. Content of the bacterial strains 4:44 and PB52 (avg $\pm$ SEM) in rotifers as measured by ELISA after 20, 40, and 60 min in suspensions of bacteria, and after transfer to seawater with added algae for 2, 4 and 24 h, indicated as 2A, 4A and 24A, respectively. The concentrations of bacteria were: $\blacktriangle$ $7.8 \times 10^6$ 4:44 ml$^{-1}$, $\bullet$ $1.7 \times 10^8$ 4:44 ml$^{-1}$, $\square$ mixture of equal numbers of 4:44 and PB52 (totally $3.5 \times 10^8$ bacteria ml$^{-1}$), and $\blacklozenge$ $3.3 \times 10^8$ PB52 ml$^{-1}$.

metanauplii tested (25 or 50 ind ml$^{-1}$) had no effect on the amount of bacteria accumulated ($P \geq 0.14$). Experiments which differed only in the density of *A. franciscana* were therefore treated as replicates. The CFU values in the water at the onset of the experiments and after 30 min of grazing were not significantly different ($P > 0.6$), indicating that the accumulation of bacteria in *A. franciscana* was not limited by the number of bacteria in suspension. The amount of bacteria accumulated in *A. franciscana* was dependent on the concentration of bacteria in the suspension (Figs. 3 and 4). PB61 was accumulated in *A. franciscana* in higher numbers than PB111, and the bacterial composition of *A. franciscana* was not affected to a large degree after incubation in suspensions of PB111 (Table 2).

The amount of accumulated bacteria present in the metanauplii after grazing for 24 h in seawater with added algae varied in the experiments with the two bacterial strains. After transfer to seawater with added algae, PB61 represented, on average, 65% of the
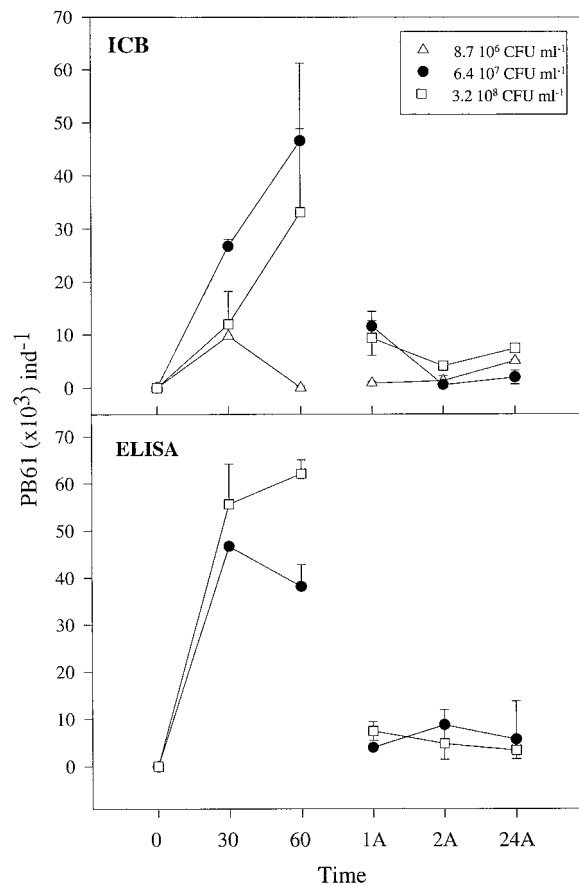


Fig. 3. Content of the strain PB61 (avg ± SEM) present in *A. franciscana* metanauplii as measured by ICB (top) and ELISA (bottom) after incubation in bacterial suspensions for 30 and 60 min (three different concentrations), and after transfer to seawater with added algae for 1, 2, and 24 h, indicated as 1A, 2A, and 24A, respectively.
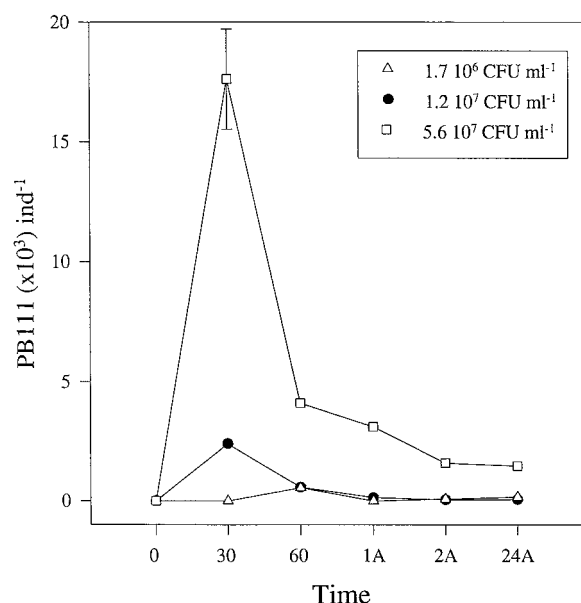
Fig. 4. Content of the strain PB111 (avg ± SEM) present in *A. franciscana* metanauplii as measured by ICB, after incubation in bacterial suspensions for 30 and 60 min (three different concentrations), and after transfer to seawater with added algae for 1, 2, and 24 h, indicated as 1A, 2A, and 24A, respectively.

total CFU (Table 2), and the total CFU was reduced to an average of $7.5 \times 10^3$ per metanauplius. In contrast, PB111 comprised $\leq 8\%$ of the total CFU, in all but one sample, and the total CFU was on average $2.8 \times 10^3$ per *A. franciscana*. After transfer to seawater with added algae, the amount of PB111 in the metanauplii, as measured by ELISA, was not significantly different ($P > 0.05$) from the detection level (results not shown).

Table 2

The bacterial strains PB61 and PB111 as a percentage (avg ± SEM) of the total CFU in *A. franciscana* after 30 and 60 min of grazing in the bacterial suspensions, and after transfer to seawater with added algae for 1, 2 and 24 h, indicated as 1A, 2A, 24A, respectively. The concentration of the bacteria strains in the bacterial suspensions (CFU ml$^{-1}$) is also given (nd: not detected)

| Concentration | PB61 | | | PB111 | | |
|---|---|---|---|---|---|---|
| | $8.7 \times 10^6$ | $6.4 \times 10^7$ | $3.2 \times 10^8$ | $1.7 \times 10^6$ | $1.2 \times 10^7$ | $5.6 \times 10^7$ |
| 30 min | 67 ± 17 | 88 ± 11 | 79 ± 21 | nd | 6 ± 3 | 9 ± 6 |
| 60 min | 37 ± 8 | 93 ± 7 | 86 ± 14 | 11 ± 9 | 4 ± 1 | 9 ± 1 |
| 1A | 44 ± 3 | 78 ± 9 | 89 ± 1 | nd | 8 ± 7 | 8 ± 6 |
| 2A | 34 ± 13 | 99 ± 4 | 100 | 4 ± 2 | 8 ± 6 | nd |
| 24A | 25 ± 4 | 88 ± 8 | 94 | 20 ± 10 | nd | 3 ± 3 |

## 4. Discussion

Bacteria were effectively accumulated in both *B. plicatilis* and *A. franciscana* within 20–30 min, which is a time period comparable to the gut passage time of the two species (Coutteau, 1991; Vadstein et al., 1993). Both *B. plicatilis* and *A. franciscana* were more efficient grazers at high bacterial densities ($\geq 5 \times 10^7$ bacteria ml$^{-1}$), where they accumulated about 10% of the total amount of bacteria present in solution during short-term incubation. These findings are in agreement with the clearance rates of *B. plicatilis* and *A. franciscana* (Vadstein et al., 1993; Makridis and Vadstein, 1999). At lower concentrations of bacteria ($< 5 \times 10^7$ bacteria ml$^{-1}$), only about 1% of the total amount of bacteria present was accumulated in *B. plicatilis*, whereas in *A. franciscana*, the number of grazed bacteria was variable. The saturation kinetics observed at high bacterial densities (ELISA results) and the constant CFU values in the water before and after grazing (*A. franciscana* experiments), indicated that the accumulation of bacteria was not limited by the number of bacteria present in the medium, but most probably by the capacity of the grazers' gut and their efficiency to filter bacteria. *B. plicatilis* accumulated an equal or higher number of bacteria than *A. franciscana* despite its smaller size. In the rotifer experiments where two bacterial strains were mixed, the interactions between the two strains had an effect on the bioencapsulation of bacteria.

The number of bacteria accumulated by *A. franciscana* was dependent on the bacterial strains used, as PB61 was grazed by *A. franciscana* more effectively than PB111 (Figs. 3 and 4). This variability is also apparent in results from earlier studies, where the values of bioencapsulated bacteria in *A. franciscana* reported range from $2.4 \times 10^3$ to $1.2 \times 10^5$ CFU per *A. franciscana* (Campbell et al., 1993; Gomez-Gil et al., 1998). The difference obtained in our study may be partly explained by the fact that the concentration of PB111 was lower than $6 \times 10^7$ CFU ml$^{-1}$, whereas PB61 was grazed at concentrations up to $3.21 \times 10^8$ CFU ml$^{-1}$.

ICB is suitable for measurements related to the delivery of live bacteria, such as probiotics, whereas ELISA is suitable in relation to the delivery of vaccines. The number of live bacteria is appropriate when it comes to measurement of doses needed to accomplish a successful colonization of the fish gut. The ICB assay was more sensitive (10 bacteria ml$^{-1}$) than ELISA ($3–5 \times 10^3$ bacteria ml$^{-1}$). The problem of low sensitivity of ELISA could be solved if live food was homogenized at a high density as it occurred in the case of rotifer samples. In the experiments with rotifers, values obtained with ELISA after 60 min of grazing were equal or higher than values obtained after 20 min of grazing, whereas the corresponding values obtained by ICB were lower after 60 min than after 20 min in three out of four cases. This indicated that a part of the bacteria was digested in the gut of the grazing animals during this time interval (Figs. 1 and 2).

The number of bacteria accumulated in the grazers in suspensions with high density of bacteria was probably too high and might induce decreased feeding by the larvae (Benavente and Gatesoupe, 1988). Grazing in seawater with added microalgae, reduced the total CFU in the live food, as the microalgae replaced the bacteria in the gut of the grazers. The percentage of the specific bacteria remained, however, at a level higher than in non-treated animals, so incubation in bacterial suspensions had influenced the

bacterial microflora of live for a longer period (Tables 1 and 2). Addition of microalgae in first feeding tanks can eliminate problems related to overloading of the live food with bacteria. Another possible solution would be to adjust the concentration of the bacterial suspensions in order to allow bioencapsulation of a relatively low number of bacteria per live food organism (around $5 \times 10^3$ bacteria ind$^{-1}$). Optimization of the bioencapsulation procedure should be done individually for each strain under specific culture conditions, as these factors may have an effect on the process. Bioencapsulation of lactic acid bacteria in rotifers fed to first feeding turbot larvae gave best results when the bacterial density was around and $1-2 \times 10^7$ bacteria ml$^{-1}$ (Gatesoupe, 1994).

In conclusion, bacteria were effectively bioencapsulated within 20–30 min in both rotifers and *A. franciscana* after incubation in the bacterial suspensions. This process is compatible with other practices in commercial aquaculture as the nutritional value of the live food does not decrease during this short period. The specific bacteria were present in the live food organisms 4–24 h after transfer to seawater with added algae. It is possible, after a short-term enrichment, to replace the opportunistic bacteria present in the live food cultures with less-aggressive bacteria, which persist as a dominant part of the bacterial flora of the live food.

## Acknowledgements

## References

Adams, A., 1991. Detection of *Vibrio parahaemolyticus* biotype *alginolyticus* in penaid shrimp using an amplified enzyme-linked immunosorbent assay. Aquaculture 93, 101–108.

Austin, B., Stuckey, L.F., Robertson, P.A.W., Effendi, I., Griffith, D.R.W., 1995. A probiotic strain of *Vibrio alginolyticus* effective in reducing diseases caused by *Aeromonas salmonicida*, *Vibrio anguillarum* and *Vibrio ordalii*. J. Fish Dis. 18, 93–96.

Benavente, G.P., Gatesoupe, F.J., 1988. Bacteria associated with cultured rotifers and Artemia are detrimental to larval turbot, *Scophthalmus maximus* L. Aquacult. Engin. 7, 289–293.

Bergh, Ø., 1995. Bacteria associated with early stages of halibut, *Hippoglossus hippoglossus* L., inhibit growth of a pathogenic *Vibrio* sp. J. Fish Dis. 18, 31–40.

Campbell, R., Adams, A., Tatner, M.F., Chair, M., Sorgeloos, P., 1993. Uptake of *Vibrio anguillarum* vaccine by *Artemia salina* as a potential oral delivery system to fish fry. Fish Shellfish Immunol. 3, 451–459.

Coutteau, P., 1991. Baker's yeast as substitute for micro-algae in the culture of filter-feeding organisms. PhD Thesis Univ. Ghent, pp. 178–181.

Dixon, B.A., Van Poucke, S.O., Chair, M., Demasque, M., Nelis, H.J., Sorgeloos, P., De Leenheer, A.P., 1995. Bioencapsulation of the antibacterial drug sarafloxacin in nauplii of the brine shrimp *Artemia franciscana*. J. Aquat. Anim. Health 7, 42–45.

Ey, P.L., Prowse, S.J., Jenkin, C.R., 1978. Isolation of pure IgG$_1$, IgG$_{2a}$ and IgG$_{2b}$ immunoglobulins from mouse serum using protein A-sepharose. Immunochemistry 15, 429–436.

Gatesoupe, F.J., 1994. Lactic acid bacteria increase the resistance of turbot larvae, *Scophthalmus maximus*, against pathogenic vibrio. Aquat. Living Resour. 7, 277–282.

Gomez-Gil, B., Herrera-Vega, M.A., Abreu-Grobois, F.A., Roque, A., 1998. Bioencapsulation of two different *Vibrio* species in nauplii of the brine shrimp (*Artemia franciscana*). Appl. Environ. Microbiol. 64, 2318–2322.

Havennar, R., Ten Brink, B., Huis in't Veld, J.H.J., 1992. Selection of strains for probiotic use. In: Fuller, R. (Ed.), Probiotics, The Scientific Basis. Chapman & Hall, London, pp. 209–224.

Hobbie, J.E., Daley, R.J., Jasper, S., 1977. Use of nuclepore filters for counting bacteria by fluorescence microscopy. Appl. Environ. Microbiol. 33, 1225–1228.

Kawai, K., Yamamoto, S., Kusuda, R., 1989. Plankton-mediated oral delivery of *Vibrio anguillarum* vaccine to juvenile ayu. Nippon Suisan Gakkaishi 55, 35–40.

Keskin, M., Keskin, M., Rosenthal, H., 1994. Pathways of bacterial contamination during egg incubation and larval rearing of turbot, *Scophthalmus maximus*. J. Appl. Ichthyol. 10, 1–9.

Kotani, H., McGarrity, G.J., 1986. Identification of mycoplasma colonies by immunobinding. J. Clin. Microbiol. 23, 783–785.

Makridis, P., Vadstein, O., 1999. Food size selectivity of *Artemia franciscana* at three developmental stages. J. Plankton Res. 21, 2191–2201.

Mohney, L.L., Lightner, D.V., Williams, R.R., 1990. Bioencapsulation of therapeutic quantities of the antibacterial Romet-30 in nauplii of the brine shrimp *Artemia* and in the nematode *Panagrellus redivivus*. J. World Aquacult. Soc. 21, 186–191.

Munro, P.D., Henderson, R.J., Barbour, A., Birkbeck, T.H., 1999. Partial decontamination of rotifers with ultraviolet radiation: the effect of changes in the bacterial load and flora of rotifers on mortalities in start-feeding larval turbot. Aquaculture 170, 229–244.

Nicolas, J.L., Robic, E., Ansquer, D., 1989. Bacterial flora associated with a trophic chain consisting of microalgae, rotifers, and turbot larvae: influence of bacteria on larval survival. Aquaculture 83, 237–248.

Olsen, Y., Reitan, K.I., Vadstein, O., 1993. Dependence of temperature on loss rates of rotifers, lipids, and ω3 fatty acids in starved *Brachionus plicatilis* cultures. Hydrobiologia 255–256, 13–20.

Olsson, J.C., Westerdahl, A., Conway, P.L., Kjelleberg, S., 1992. Intestinal colonization potential of turbot (*Scophthalmus maximus*)- and dab (*Limanda limanda*)-associated bacteria with inhibitory effects against *Vibrio anguillarum*. Appl. Environ. Microbiol. 58, 551–556.

Porter, K.G., Feig, Y.S., 1981. The use of DAPI for identification and counting of aquatic microflora. Limnol. Oceanogr. 25, 943–948.

Reitan, K.I., Rainuzzo, J.R., Øie, G., Olsen, Y., 1993. Nutritional effects of algal addition in first feeding tanks of turbot (*Scophthalmus maximus* L.) larvae. Aquaculture 118, 257–275.

Ringø, E., Birkbeck, T.H., Munro, P.D., Vadstein, O., Hjelmeland, K., 1996. The effect of early exposure to *Vibrio pelagius* on the aerobic bacterial flora of turbot, *Scophthalmus maximus* (L.) larvae. J. Appl. Bacteriol. 81, 207–211.

Ringø, E., Sinclair, P.D., Birkbeck, H., Barbour, A., 1992. Production of eicosapentaenoic acid (20:5 n-3) by *Vibrio pelagius* isolated from turbot (*Scophthalmus maximus* (L.)) larvae. Appl. Environ. Microbiol. 58, 3777–3778.

Skjermo, J., Vadstein, O., 1993. Characterization of the bacterial flora of mass cultivated *Brachionus plicatilis*. Hydrobiologia 255–256, 185–191.

Skjermo, J., Vadstein, O., 1999. Techniques for microbial control in the intensive rearing of marine fish larvae. Aquaculture 177, 333–343.

Sugita, H., Miyajima, C., Deguchi, Y., 1991. The vitamin B$_{12}$-producing ability of the intestinal microflora of freshwater fish. Aquaculture 92, 267–276.

Vadstein, O., Øie, G., Olsen, Y., 1993. Particle size dependent feeding by the rotifer *Brachionus plicatilis*. Hydrobiologia 255–256, 261–267.

Watanabe, T., Kitajima, C., Fujita, S., 1983. Nutritional values of live organisms used in Japan for mass propagation of fish: a review. Aquaculture 34, 115–143.

# Paper V

# Paper VI

**An <u>in vivo</u> test of probiotic candidates from the dominant and the antagonistic bacterial flora of cod larvae (<u>Gadhus morhua</u> L.). Evaluation of colonization efficiency and viability of larvae.**

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**Abstract**

A first feeding experiment with Atlantic cod larvae was conducted to evaluate in vivo effects of probiotic candidate bacteria. The experiment included three conditions: 1. Control with no addition. 2. Addition of a mixture of three bacteria from the dominant bacterial flora of cod larvae. 3. Addition of a mixture of two bacteria from the antagonistic bacterial flora of cod larvae. The five candidate bacteria were isolated from healthy cod larvae, and characterized with respect to antagonistic activity, adhesion to and growth in mucus, extracellular enzyme production, haemolytic activity, and bile tolerance. The candidate bacteria were added directly to the rearing water on the day of hatching, and bioencapsulated in rotifers that were fed to the larvae. The culture independent method terminal restriction fragment length polymorphism (T-RFLP) was used to detect the added bacteria in water, rotifers and larvae. The CFU in the rearing water increased 76 and 164% in treated tanks compared to the control after the direct addition of the bacteria on the day of hatching. However, only one of the added bacteria was detected in the water by T-RFLP. The added bacteria were successfully bioencapsulated in rotifers, but at lower percentages than studies using culture based methods for detecting the bacteria. A non-significant increase of the added bacteria was detected in rotifers taken from the rearing tanks on day 5, whereas the added bacteria were not detected in the rotifers on later stages. The added bacteria were not detected in cod larvae, implying low colonisation potential of the added bacteria. This was in contrast to the in vitro test results of the added bacteria, where one of the bacteria showed high adhesiveness to mucus and two of the bacteria showed high growth rates in mucus. However, in individual larvae on day 17, the T-RFLP data showed individual differences within the same rearing tanks, both regarding TRFs corresponding to the

added and to other bacteria. Although larvae in treated tanks did not perform better than larvae in the control tanks, high growth rates were obtained in the rotifer feeding period, and high survival was experienced in the weaning phase. Thus the lack of positive effects of the probiotic candidates may have been due to the fact that the potential for further improvement was low.

**Keywords**

T-RFLP, Probiotic, Atlantic cod (Gadhus morhua L.), Dominant bacterial flora, Antagonistic bacterial flora, First-feeding.

**Introduction**

The intensive rearing conditions during production of Atlantic cod larvae (Gadhus morhua L.) provide an environment suited for opportunistic bacteria (Skjermo et al. , 1997), and the production of larvae has been hampered by low and unpredictable growth and survival during first-feeding. Several strategies have been proposed to improve the control of microbial conditions in the production of marine fish larvae (Skjermo and Vadstein, 1999). The use of microbially matured rearing water is a strategy that reduces the number of opportunistic bacteria (Skjermo et al. , 1997) and may enhance growth of the larvae (Salvesen et al. , 1999), while the use of water recirculating systems stabilizes the microbial community in the rearing water compared to flow-through systems (Attramadal, 2004). Reduction or exchange of the bacterial load of live feed (Munro et al. , 1999; Makridis et al. , 2000a; Olsen et al. , 2000), addition of immunostimulants to boost the non-specific immune system (Vadstein, 1997; Conceicao et al. , 2001; Skjermo and Bergh, 2004) and the use of probiotics (Gatesoupe,

1999; Skjermo and Vadstein, 1999; Irianto and Austin, 2002) are other promising strategies that have been applied.

Probiotics was defined by Gram and Ringø (2005) as "live microbial cultures added to feed or environment (water) to increase viability (survival) of the host". In most studies, probiotic candidates for marine fish larvae are chosen because they show in vitro inhibitory activity against a target pathogen, although it has not been shown that in vitro antagonism is actually effective in vivo (Atlas, 1999). The dominant bacterial flora from marine larvae in well performing rearing groups, have more rarely been evaluated as probiotic candidates (Skjermo and Vadstein, 1999; Huys et al. , 2001). It is assumed that bacteria showing a dominant colonization of the intestinal mucus are capable of excluding pathogens from the adhesion sites of the gut wall through competition (Verschuere et al. , 2000). Bacteria are often screened for several in vitro properties, before they are proposed as probiotic candidates and tested in vivo. It is important to know how the in vitro properties are reflected under in vivo conditions to enable efficient selection of probiotic candidate bacteria. The bacterial colonisation potential is regarded as an important property for probionts, and bacterial isolates can be screened in vitro for this property (Vine et al. , 2004; Fjellheim et al. in prep. c). Colonisation can be defined as the ability of an organism to establish and remain in a niche after exogenous supply has stopped (Gram and Ringø, 2005). The colonisation resistance of the existing bacterial flora, makes colonisation by introduced bacteria more difficult. Thus, colonisation by added bacteria can most easily be done in the early stages of host colonisation.

Terminal restriction fragment length polymorphism (T-RFLP) is a culture independent molecular method that has been used to characterize the microbial composition in several habitats (Liu et al. , 1997; Osborn et al. , 2000). DNA from a sample is used as the template for the PCR and at least one of the primers is labelled with a fluorescent dye when running T-RFLP. Amplified products are digested with one or more restriction enzymes separately and the size of the fluorescent terminal fragment is determined. DNA extraction efficiency and PCR bias, as well as the choice of primers and restriction enzymes, can influence the T-RFLP results (Osborn et al. , 2000).

In this study we wanted to evaluate probiotic candidate bacteria in a first-feeding experiment with cod larvae. The probiotic candidates were found using two different selection strategies, i.e. from the dominant- and the antagonistic- intestinal bacterial flora of cod larvae (Fjellheim et al. in prep. a and b). The bacteria were selected from a library of nearly 500 bacterial isolates, based on several _in vitro_ characteristics (Fjellheim et al. in prep. c). In the first feeding experiment we monitored the bacterial composition in the rearing water, the live feed and the cod larvae using traditional methods and T-RFLP. In addition, the presence of the added bacteria was detected by T-RFLP (Jernberg et al. , 2005), and T-RFLP was used to quantify the relative abundance of the added bacteria.

**Materials and methods**

*First feeding experiment*

The first feeding experiment included two experimental treatments (D and A) and a control (C). Treatment D received three probiotic candidates that had previously been isolated from the dominant intestinal bacterial flora of Atlantic cod larvae (Fjellheim et al. in prep. a). Treatment A received two probiotic candidates that had been isolated after screening for antagonistic isolates among the intestinal bacterial flora in cod larvae (Fjellheim et al. in prep. b). Three replicate rearing tanks were used for the two treatments and the control.

The seawater was sandfiltered, run through a maturation unit (Skjermo et al. , 1997) and vacuum aerated before entering the 160-l conical rearing tanks. The cod eggs were disinfected for 8 min in 1600 mg glutaraldehyde $l^{-1}$ (Salvesen and Vadstein, 1995), distributed in the tanks (40 eggs $l^{-1}$) and kept in darkness until two days after hatching. The water temperature was gradually increased from 7°C at the start of the experiment to 12°C on day 7 after hatching, and then kept at this temperature. The water exchange rate was initially 1 exchange $day^{-1}$ and increased to 2 $day^{-1}$ on day 4, 3 $day^{-1}$ on day 12, 5 $day^{-1}$ on day 18 and maximum exchange rate of 8 $day^{-1}$ on day 21 after hatching. The tanks were aerated moderately with airstones.

Microalgae (Isochrysis galbana) were added on the day of hatching to a final concentration of 1 mg algal C $l^{-1}$, and maintained at this concentration by addition of algae once daily until day 3 after hatching and twice daily thereafter. The microalgae

were cultivated in 200-l cylindrical tanks with F/2 medium (Guillard and Ryther, 1962) prepared from sandfiltered seawater. The algal cultures were grown semi-continuously (30% dilution day$^{-1}$) and illuminated with fluorescent tubes.

The rotifers (<u>Brachionus</u> sp. Nevada, SINTEF strain) (Gomez et al. , 2002; Papakostas et al. , 2006) were cultured in 250-l conical tanks in brackish water (20‰) at 20°C and fed fresh baker`s yeast (1.2 µg ind$^{-1}$), marol E (7.5% of yeast weight) and Chlorella (0.5 ml/mill rotifers). The cultures were grown semi-continuously by 20% dilution day$^{-1}$. Before use, the rotifers were harvested and washed with brackish water (20‰, 17°C) on a plankton net (70 µm). Rotifers  were added to the rearing tanks at a final density of 5000 rotifers liter$^{-1}$ and maintained at this concentration by addition of rotifers two or three times a day. The rotifer density in the tanks was monitored by an automatic rotifer counter (Alver et al. in prep.).

Rotifers and formulated dry feed (Gemma micro 150) were co-fed on days 18 to 21 after hatching, and the larvae were only fed dry feed thereafter. This was an early transition to dry feed compared to other rearing protocols (Brown et al. , 2003; Korsnes et al. , 2006), and was considered to be a stress test for the larvae. Dead larvae and debris was removed from the tanks 1, 2, 6, 9, 12, 14 and 18 days after hatching, and every day thereafter. Dead larvae were counted daily from the introduction of dry feed and to the end of the experiment. At the termination of the experiment all live larvae were counted.

*Probiotic candidates*

The three probiotic candidates that were added in treatment D (ID3-10, ND2-7 and RD5-30), were isolated from the dominant intestinal bacterial flora in Atlantic cod larvae from three different well-performing rearing groups (Fjellheim et al. in prep. a). The dominant candidates had 16S rDNA similarity to Microbacterium sp. (ID3-10 and ND2-7) and to Vibrio gallicus (RD5-30). The two probiotic candidates that were added in treatment A (RA4-1 and RA7-14), were isolated after screening the intestinal bacterial flora in cod larvae for antagonism against Listonella anguillarum (strain HI 610). Both isolates were from the same rearing group and had DNA sequence similarity to Roseobacter sp. (RA4-1) and to Pseudoalteromonas sp. (RA7-14) (Fjellheim et al. in prep. b). These probiotic candidates were selected from an initial pool of 370 dominant and 98 antagonistic isolates. The isolates were chosen after in vitro evaluation of antagonism against four pathogens and three dominant isolates, adhesion to mucus, growth rate in mucus, production of extracelluar enzymes, haemolytic activity, and fish bile resistance. In addition a small scale in vivo test with exposure of cod yolk sac stage larvae was performed to evaluate probiotic and detrimental effects of the isolates on the larvae (Fjellheim et al. in prep. c). Observed TRFs were found by running pure cultures of the five bacteria through the T-RFLP analysis, while the expected TRFs were calculated from the DNA sequence. The properties of these five isolates are given in Table 1.

*Addition of probiotic candidates in water and in rotifers*

The probiotic candidate bacteria were grown in light for 2-3 days at 20°C in M-65 broth (0.5 g peptone, 0.5 g tryptone, 0.5 g yeast extract, 800 ml seawater, 200 ml distilled

water) before they were added to the water or bioencapsulated in the rotifers. The density of bacteria was determined by measuring absorbance (660 nm) and converted to cells ml$^{-1}$ by the use of strain specific calibration. The probiotic candidates were added directly to the rearing water on the day of hatching to a final concentration of $10^5$ bacteria ml$^{-1}$ (i.e $3.3 \times 10^4$ of each candidate in treatment D and $5 \times 10^4$ of each candidate in treatment A). This was verified by plate counts. The probiotic candidates were bioencapsulated in rotifers and given to cod larvae on day 2, 3, 4, 6, 8, 11 and 16 after hatching. Suspensions of bacteria were prepared in seawater for the two treatments with a final concentration of approximately $10^8$ bacteria ml$^{-1}$. Equal numbers of the three dominant candidates were used in one suspension and likewise in the antagonistic suspension. The rotifers (1000 ind ml$^{-1}$) grazed in the bacterial suspensions for 30 min with aeration, were rinsed with brackish water (20‰) for 5 min, and then added to the rearing tanks. Rotifers fed to control tanks were treated in the same way, but bacteria were not added.

*Sampling procedures*

The number of colony forming units (CFU) on M-65 agar (0.5 g peptone, 0.5 g tryptone, 0.5 g yeast extract, 15 g agar, 800 ml seawater, 200 ml distilled water) was determined for water, rotifers and for cod larvae. Agar plates were incubated at 15°C and counted after 2 and 20 days to determine the percent of visible colonies on day 2 (PV2) (Salvesen and Vadstein, 2000). Selected agar plates from larvae were replica-plated onto blood-agar (cattle blood, added 2% NaCl) to determine the proportion of haemolytic bacteria. Water samples were taken from all tanks on day 0 to verify that the probiotic candidates were added to the intended concentrations. Samples of rotifers

were taken from all tanks on day 5 and 17 after hatching. At each sampling, the rotifers in approximately 400 ml of tank water were rinsed and concentrated in 40 ml of autoclaved seawater. The number of rotifers in this suspension was determined by counting the samples in a microscope. Then the rotifers were concentrated to 1 ml samples, homogenized, diluted, and plated on M-65 agar plates. Samples of cod larvae were also taken 5 and 17 days after hatching. At each sampling 10 larvae from each tank were starved for 3-4 hours, anaesthetized (30 sec, metomidate 100 mg/l), surface disinfected (30 sec, 0.1% benzalkoniumcloride) and washed twice (2 x 60 sec) in sterile 80% seawater. The larvae were then homogenized in 1 ml sterile 80% seawater and serial dilutions of the homogenate were plated in duplicate on M-65 agar plates.

DNA samples of bacteria were taken from water, rotifers and cod larvae. Water samples were taken on day 0, 5, 10, 17, 24 and 31 after hatching by prefiltering rearing water to remove rotifers, and then filtering 50 ml rearing water through 0.2 µm filters (Dynagard, Microgon Inc.). Samples of rotifers were taken from all tanks on day 5, 10 and 17 after hatching. At each sampling, the rotifers in approximately 400 ml of tank water were rinsed using 200 ml autoclaved seawater and 50 ml DNA free water (Eppendorf). The rotifers were concentrated in 40 ml of DNA free water. The rotifer density in the suspension was determined by counting the samples in a microscope. Then the rotifers in the suspension were separated from the water using a 0.2 µm filter (Dynagard). Samples of 10 cod larvae were taken from all tanks on day 5, 10, 14, 17, 24 and 31 after hatching. The larvae were starved for 3-4 hours, anaesthetized (30 sec, metomidate 100 mg/l), washed with distilled and with DNA free water, and then they were transferred to

180 µl ATL buffer (provided in the Qiagen DNeasy Tissue Kit). All samples were stored at -20°C until further processing.

*DNA extraction*

DNA was extracted using a DNA purification kit (Qiagen, DNeasy Tissue Kit). Filter samples (water and rotifers) were centrifuged to remove excess water, prior to DNA extraction. The ATL buffer (180 µl) and the proteinase K (20 µl) were incubated in the filters, and the content was centrifuged into eppendorf-tubes. The larvae were stored in the ATL buffer in eppendorf-tubes and thus proteinase K (20 µl) was added directly, and the mix was incubated for 1-2 hours at 55°C to lyse the samples. From there on the extraction followed the procedure given by the manufacturer. Water and rotifer samples were eluted twice in 50 µl Buffer AE and samples with larvae were eluted once in 100 µl of Buffer AE. DNA content in the extracts was measured with a NanoDrop ® spectrophotometer.

*Real Time PCR and T-RFLP*

The target region of the 16S rDNA was amplified in the real time PCR assay using the fluorescence-labeled forward primer 8f (5`-FAM-AGA GTT TGA TC(AC) TGG CTC AG – 3`) and the reverse primer 517r (5`-ATT ACC GCG GCT GCT GG – 3`). The 25 µl reaction mixture included Power SYBR®Green PCR Master mix (Applied Biosystems), primers (0.2 µM each), 7.5 µg bovine serum albumin (Sigma) and 5 µl DNA template. PCR amplification was performed with the 7500 Real Time PCR System (Applied Biosystems). To keep the fluorescent detection non-selective, we used the non-specific intercalating dye SYBR Green I as the reporter dye. The PCR

amplifications were performed using an initial denaturation at 95°C for 10 min followed by variable number of cycles of denaturation at 95°C for 15 sec, and primer annealing and extension at 60°C for 1 min. The samples were run the required number of cycles to end the PCR in the late logarithmic DNA amplification phase (17 to 31 cycles). A negative control was included in each PCR run.

The PCR products were digested with the restriction enzyme AluI at 37°C for one hour. Some samples were also digested with Hinf I (data not shown). The digestion mixture (30 µl) contained 5 µl PCR product, 10 U AluI and 10 × buffer. Digested PCR products were precipitated with ethanol (96%) and 3 M NaAc and the DNA pellet was then washed with 70% ethanol. The pellet was air dried at 50°C and stored at -20°C. The samples were prepared for analysis by adding 12 µl of formamide and 0.15 µl of Genescan$^{TM}$ 500 LIZ$^{TM}$ Size standard (Applied Biosystems) to the digested PCR product. Samples were denatured at 95°C for 6 min and then rapidly chilled on ice. The lengths of TRFs were determined with an ABI Prism 3130xl Genetic Analyzer (Applied Biosystems), and the peak area data were standardized by the variable percentage threshold method (Osborne et al. , 2006). The divisor that resulted in the weakest relationship between the number of peaks remaining and the initial total area was used for each of the sample types (water, enriched rotifers, rotifers in tanks, and larvae).

*Dry weight*

The growth of the larvae was determined by measuring dry weight. Twelve larvae from each tank were washed in freshwater, and transferred to separate tin capsules on day 0, 2, 5, 10, 17, 24 and 31 after hatching. The capsules were dried at 60°C for 3-5 days

before the dry weight of larvae was determined. The specific growth rate (SGR) was calculated as:

$$SGR = \ln(W_t/W_0) / t$$

Where $W_t$ is mean final dry weight, $W_0$ is initial dry weight, and t is the time between initial and final sampling. Percentage daily specific growth rate was calculated from SGR as:

$$\% SGR = (\exp^{SGR} - 1) \times 100$$

*Statistics*

ANOVA and Tukey´s multiple comparison test were used to compare treatments regarding weight, survival and the presence of added bacteria in water, rotifers and larvae (T-RFLP data). Prior to the statistical tests, data in percentages were arcsin-transformed and data on the dry weight of the larvae were log transformed to stabilize variation. Differences in the total bacterial flora (T-RFLP data) within and between the different groups was tested using the vegan package in the R project statistics (Clarke, 1993; Oksanen et al. , 2005; R Development Core Team, 2005).

**Results**

*Rearing water*

The five probiotic candidate bacteria were added directly to the rearing water on the day of hatching. After addition of the bacteria, the CFU increased significantly (Table 2) in tanks receiving treatment A compared to the control tanks (p=0.002). The CFU in tanks receiving treatment D increased, but not significantly, compared to the control tanks (p=0.059). The percentage of fast-growing colonies visible on day 2 on agar (PV2), was not significantly different between treatments on day 0 (p=0.073). The T-RFLP data showed that the TRFs corresponding to the antagonistic probiotic candidate RA4-1 increased significantly (p<0.001) in the rearing water on day 0 (Table 3). In addition, the probiotic candidates RA7-14 and RD5-30 were detected at higher levels compared to the control on day 0, although the increase was not significant. However, the total bacterial flora detected by T-RFLP was significantly different between treatments and the control on day 0 (p=0.01). Other bacteria in the rearing system had the same TRFs as the added probiotic candidates. Treated tanks therefore had to show increased levels compared to the control to demonstrate the presence of the added bacteria. As the water exchange rate was increased in the rearing tanks, the presence of the added bacteria could not be detected at significantly higher levels in treated tanks, compared to the control. In addition, the total bacterial flora of the water was not affected by the addition of probiotic candidates with higher flow through rates (day 5: p=0.238 and day 10: p=0.589).

*Rotifers*

The five probiotic candidates were bioencapsulated in rotifers to change the bacterial flora of the rotifers and to facilitate transfer to the larvae. The level of added bacteria in rotifers after bioencapsulation, but before the rotifers were fed to the larvae was checked twice (Table 4). In rotifers receiving treatment D, the TRFs corresponding to the two Microbacterium strains was detected at higher levels on day 4, but only slightly higher on day 11, compared to the control. The third probiotic candidate that was added in the dominant treatment (Vibrio) constituted about 5% of the total peak area in the T-RFLP data on both samplings, while it was not found in the control. In rotifers receiving treatment A, a higher level of the TRFs corresponding to the probiotic candidate RA7-14 (Pseudoalteromonas) was found on day 11, but the level was not higher on day 4, compared to the control. The TRF corresponding to the Roseobacter, also added in the antagonistic treatment, was detected at higher levels on both samplings compared to the control. Thus the percentage of added bacteria ranged from 6 to 53% in the rotifers after the bioencapsulation procedure. Moreover, variable levels of added bacteria were found in the rotifers between the two samplings. High numbers of cultivable bacteria were found in rotifers from the rearing water on day 5 compared to day 17 (Table 2), but the numbers of bacteria in rotifers were not significantly different between treatments. Moreover, the percentage of fast-growing bacteria (PV2) was lower in rotifers on day 17 compared to day 5. T-RFLP data showed that the TRFs corresponding to the added bacteria were detected at higher levels, although not significantly higher, in treated rotifers on day 5 compared to the control (Table 5). The added bacteria were not detected in rotifers from the rearing water on day 10 and 17.

*Cod larvae*

The average CFU in cod larvae varied between the rearing tanks both on day 5 and day 17, as shown by the high standard deviations (Table 2). The percentage of fast-growing bacteria (PV2) was reduced in larvae from treatment D and the control, during the rotifer period, whereas in larvae from treatment A the PV2 value was similar. The percentage of haemolytic bacteria was reduced in larvae from all tanks, whereas the percentage of Vibrio was similar, based on comparisons of cod larvae on day 5 and 17. We did not detect significant levels of the added bacteria in larvae with the culture independent method T-RFLP for pooled samples of larvae (Table 6). Moreover, the composition of the total bacterial flora was not significantly different when comparing treated larvae and larvae in the control on day 5 (p=0.417), day 10 (p=0.514) or day 31 (p=0.187). However, the TRFs corresponding to the added Vibrio (RD5-30) was found at a low level in treated larvae on day 17 after hatching, whereas it was not found at all in the control. In addition to pooled samples of larvae, four individual cod larvae from each tank were analyzed with T-RFLP on day 17 after hatching (Figure 1). These T-RFLP data showed large individual differences within the same rearing tanks, both regarding TRFs corresponding to the added bacteria and other bacteria. In two of the larvae from treatment D, the TRFs corresponding to the added Vibrio (187 ± 1 bp) constituted 3.4 and 1.5%, whereas it was not found in any other larvae. Also, the share of the TRFs corresponding to the two Microbacterium strains (71 ± 1 bp) was highly variable (0.0-32.5%) in larvae from treatment D. In the individual larvae receiving treatment A, we also found variable levels of the TRFs corresponding to the added Roseobacter (249 ± 1 bp: 1.6-8.4%) and the added Pseudoalteromonas (239 ± 1 bp: 2.4-47.9%). The bacterial flora was significantly different between the 12 individual larvae

from the three tanks receiving treatment A (p=0.001) and treatment D (p=0.047) according to T-RFLP data, whereas significant differences were not found between the larvae in the control tanks (p=0.578). When the bacterial flora from individual larvae was compared between the two treatments and the control, despite the differences within treatments, significant differences were found (p<0.001).

*Growth and survival*

The increase in individual dry weight of cod larvae was high in all tanks, and the dry weight ranged from 497 to 701 µg larva$^{-1}$ after the rotifer feeding period (Table 7). No significant differences were found between treatments or the control on day 5 (p=0.142), day 10 (p=0.900) or day 31 (p=0.861). However, on day 17 larvae from treatment A were significantly smaller than larvae from treatment D and from the control (p<0.05). Relatively high survival was recorded during the weaning phase (41.5 - 80.1%), and no significant differences were observed between treatments and the control on any days (Figure 2). However, cod larvae in one of the tanks within treatment A had increased mortality from day 12. Dead larvae and rotifers turned pink within 12 hours in this tank.

**Discussion**

The culture independent method T-RFLP (Liu et al. , 1997; Osborn et al. , 2000) was used to characterize the bacterial flora in water, rotifers and larvae. In addition, the added bacteria were detected with T-RFLP (Jernberg et al. , 2005). As DNA samples for T-RFLP analysis were amplified with real-time PCR in order to detect the late exponential amplification phase for each sample, the number of PCR cycles was reduced to a minimum, and the risk for changing the ratios between different DNA

templates minimized (Suzuki and Giovannoni, 1996; Kanagawa, 2003). The added bacteria were detected by calculating the percentage of TRFs corresponding to these bacteria in treated tanks compared to the control tanks. The TRFs of the added bacteria was defined as the observed TRF ± 1 bp. This variability was included because variation between observed TRF lengths has been found in replicate runs of the same sample (Kaplan and Kitts, 2003).

Atlantic cod and other marine larvae drink water after hatching to keep the osmotic balance (Mangor Jensen and Adoff, 1987; Reitan et al. , 1998). At this stage the clearance rate of bacteria is considerably higher than the drinking rate, indicating selective uptake of bacteria (Reitan et al. , 1998). Bacteria added to the rearing water have been shown to colonize turbot larvae (Ringø et al. , 1996; Ringø and Vadstein, 1998; Makridis et al. , 2000b). In the present study we added the probiotic candidates to the rearing water on the day of hatching to facilitate an early colonization of the larvae. The CFU counts confirmed higher levels of bacteria in the water after addition of the probiotic candidates (Table 2). The T-RFLP data indicate a significant increase in the TRFs corresponding to one of the added bacteria (Roseobacter, treatment A) on day 0, but elevated levels were also detected of the added Pseudoalteromonas (treatment A) and the added Vibrio (treatment D) in the water. The cultivability of bacteria in seawater is often <1% (Nair et al. , 1994; Attramadal, 2004), thus even if the added bacteria constituted 50% of the cultivable bacteria, this might correspond to <0.5% of the total number of bacteria. This fact might explain why we had difficulties in detecting the added bacteria on day 0 when using the culture independent method.

The T-RFLP data for rotifers after bioencapsulation of bacteria, showed that the bioencapuslation efficiency varied between the five added bacterial strains as well as between the two samplings (Table 4). The percentage of the added bacteria in the rotifers was considerably lower than observed earlier (Makridis et al. , 2000a). The difference could be explained by the different detection methods used, i.e. culture based vs. non-culture based method. Also, a more thorough washing procedure was used in the present study, which might remove more of the surface associated bacteria. A large percentage of bacteria are associated to the outside of rotifers (Munro et al. , 1993). Finally, different bacteria were used, and species differences in the encapsulation efficiency have been observed (Makridis et al. , 2000a). These observations indicate that the bioencapsulation efficiency of bacteria in rotifers should be tested when using new bacterial species and new mixtures. The fact that significant incorporation of bacteria was observed in rotifer cultures but not in rotifers from the first feeding tanks, emphasize the importance of rotifers being eaten shortly after transfer to rearing tanks if efficient transfer of added bacteria to larvae is to be obtained (Makridis et al. , 2000a).

The added bacteria were not detected by T-RFLP in treated- compared to the control-larvae in the pooled samples on any days (Table 6). The detection limit for the added bacteria was not determined, but the fact that other bacteria in the system had the same TRFs as the added bacteria, makes detection of low levels of the added bacteria difficult with this method. Thus it seems as if the colonisation potential in vivo of these five probiotic candidates was low. However, the in vitro tests on adhesion to mucus and growth in mucus (Fjellheim et al. in prep. c) indicate that at least one of the added bacteria (Roseobacter) had high adhesion to mucus (20%) and that the two bacteria

Pseudoalteromonas and Vibrio had high growth rates in mucus (0.097 and 0.075 h$^{-1}$, respectively).Thus, the in vitro properties of the added bacteria could not be verified under in vivo conditions. Bacteria with antagonistic activity in vitro, were added in treatment A. We could, however, not detect a decrease in the bacterial diversity or composition in the larvae because of this addition. The bacteria were added to the rearing water on the day of hatching to facilitate early colonisation of the larvae, but our results indicate that this might be too late. Possibly, the tank water should be conditioned with the probiotic candidates even earlier and at higher density. The percentage of haemolytic bacteria in the cod larvae was lower both on day 5 and 17, compared to turbot (Salvesen et al. , 1999). However, a similar decrease in the percentage of haemolytic bacteria was observed in both studies when the water flow-through was increased. None of the added probiotic candidates were haemolytic, as haemolysis is often associated to pathogenic bacteria (Austin et al. , 2005). However, the addition of the probiotic candidate bacteria did not lead to a significant decrease in the percentage of haemolytic bacteria, compared to the control.

The total bacterial flora of individual cod larvae was analyzed with T-RFLP on day 17 after hatching (Figure 1). Individual differences were found between larvae from the same tanks, which is in agreement with the individual differences found in the dominant bacterial flora of cod larvae from different hatcheries (Fjellheim et al. in prep. a). This suggests that the colonisation potential of bacteria varies between individuals. These individual differences might be due to stochastic factors occurring during colonisation (Verschuere et al. , 1997) or to genetic differences between individuals (Vaahtovuo et

al. , 2003). These individual differences complicate the task of selecting appropriate probiotic candidates.

The development in the weight and the survival of larvae in the weaning phase were not significantly different in the treated tanks, compared to the control. Thus, the added bacteria did not improve viability at this level. However, larvae in both the treated tanks and the control had high growth rates during the rotifer feeding period compared to previous studies (Browman et al. , 2006; Park et al. , 2006). Moreover, in a study performed in the same rearing system as the present study (Skjermo et al. accepted, 2006), the dry weight of larvae was $\pm$ 250 µg on day 17, compared to 497 to 701 µg on day 17 in the present study. In addition, a relatively high survival of 41.5 - 80.1% was obtained in the weaning phase in the present study. The survival of cod larvae in the weaning phase (day 18 – 30) in the study performed by Skjermo et al. (accepted 2006) was $\pm$ 40%. Thus, both growth rate and survival indicate high viability for both treated and control larvae. This implies that the potential for further improvement might have been low in the present study. In addition to improving growth and survival, probiotics can improve stress tolerance and non-specific immune responses (Taoka et al. , 2006). The transition from live feed to dry feed was done early in the present study (day 18 to day 21) to work as a stress test. The growth rates of the larvae were considerably lowered after transition to dry feed (from 20-30 %SGR day$^{-1}$ to 2-6 %SGR day$^{-1}$), but a relatively high survival was maintained. However, the results indicated no differences in stress tolerance between treated and control larvae.

The probiotic candidates that were used in the present study were selected by two different screening strategies and resulted in enhanced survival of yolk sac stages of cod (Fjellheim et al. in prep. c). We can not, based on the results obtained in the first feeding experiment, recommend one of the selection strategies over the other. Probiotic bacteria have to compete in a complex and changing ecological environment, and mixtures of bacterial strains with diverse properties could therefore have better chances of success than the addition of single strains of bacteria (Freter, 1992). Also, the individual differences observed in the bacterial flora of cod larvae (Fjellheim et al. in prep. a and b), could imply that a mixture of several bacterial isolates has a higher probability of colonizing larvae at the individual level with positive effects on viability. Synergistic effects can be the implication when mixtures of bacteria are used, with the result that the positive effect of the mixture exceeds the summed effect of the individual strains (Douillet, 2000). Several characteristics are thought of as important for probiotic bacteria. The bacteria added in this study were evaluated in vitro according to antagonism, adhesion to mucus, growth in mucus, and other criteria (Table 1). In these in vitro tests, the five added bacteria showed profound differences in adhesive abilities to mucus and in growth rates in cod mucus. However, the differences in the in vitro tests do not seem to be directly transferable to in vivo conditions, as none of the added isolates were detected at substantial levels in the bacterial flora of larvae. This emphasises the importance of establishing in vitro methods that can be used to predict in vivo properties of bacteria with certainty.

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**References**

Alver, M.O., Tennøy, T., Alfredsen, J.A., Øie, G. (in prep.) Automatic measurement of rotifer Brachionus plicatilis densities in first feeding tanks.

Atlas, R.M. (1999) Probiotics - snake oil for the new millennium? Environ. Microbiol. 1:377-382.

Attramadal, K.J.K. (2004) Water quality and microbial environment in a flow through and a recirculating system for intensive rearing of cod larvae (Gadhus morhua L.). Master thesis in Aquaculture and Marine Resources. Norwegian University of Science and Technology (NTNU).

Austin, B., Austin, D., Sutherland, R., Thompson, F., Swings, J. (2005) Pathogenicity of vibrios to rainbow trout (Oncorhynchus mykiss, Walbaum) and Artemia nauplii. Environ. Microbiol. 7:1488-1495.

Browman, H.I., Skiftesvik, A.B., Kuhn, P. (2006) The relationship between ultraviolet and polarized light and growth rate in the early larval stages of turbot (Scophtalmus maximus), Atlantic cod (Gadus morhua) and Atlantic herring

(Clupea harengus) reared in intensive culture conditions. Aquaculture 256:296-301.

Brown, J.A., Minkoff, G., Puvanendran, V. (2003) Larviculture of Atlantic cod (Gadus morhua): progress, protocols and problems. Aquaculture 227:357-372.

Clarke, K.R. (1993) Nonparametric multivariate analyses of changes in community structure. Aust. J. Ecol. 18:117-143.

Conceicao, L.E.C., Skjermo, J., Skjåk-Bræk, G., Verreth, J.A.J. (2001) Effect of an immunostimulating alginate on protein turnover of turbot (Scophthalmus maximus L.) larvae. Fish Physiol. Biochem. 24:207-212.

Douillet, P.A. (2000) Bacterial additives that consistently enhance rotifer growth under synxenic culture conditions 2. Use of single and multiple bacterial probiotics. Aquaculture 182:241-248.

Fjellheim, A.J., Playfoot, K.J., Skjermo, J. Vadstein, O. (in prep. a) Large inter-individual variation in the dominant intestinal bacterial flora of reared cod larvae.

Fjellheim, A.J., Playfoot, K.J., Skjermo, J. Vadstein, O. (in prep. b) The antagonistic bacterial flora of intensively reared cod larvae is dominated by Vibrio.

Fjellheim, A.J, Klinkenberg, G., Skjermo, J., Aasen, I.M.,Vadstein, O (in prep. c) A comparison of candidate probiotic bacteria selected by two different screening strategies.

Freter, R. (1992) Factors affecting the microecology of the gut. In: Fuller, R. (Ed.) Probiotics. The scientific basis. Chapman & Hall, London, pp 111-144.

Gatesoupe, F.J. (1999) The use of probiotics in aquaculture. Aquaculture 180:147-165.

Gomez, A., Serra, M., Carvalho, G.R., Lunt, D.H. (2002) Speciation in ancient cryptic species complexes: Evidence from the molecular phylogeny of <u>Brachionus plicatilis</u> (Rotifera). Evolution; Int. J. Org. Evol. 56:1431-1444.

Gram, L., Ringø, E. (2005) Prospects of fish probiotics. In: Holzapfel, W., Naughton, P. (Eds.) Microbial ecology of growing animals. Elsevier.

Guillard, R.R., Ryther, J.H. (1962) Studies of marine planktonic diatoms .1. <u>Cyclotella nana</u> hustedt, and <u>Detonula confervacea</u> (cleve) gran. Can. J. Microbiol. 8:229-239.

Huys, L., Dhert, P., Robles, R., Ollevier, F., Sorgeloos, P., Swings, J. (2001) Search for beneficial bacterial strains for turbot (<u>Scophthalmus maximus</u> L.) larviculture. Aquaculture 193:25-37.

Irianto, A., Austin, B. (2002) Probiotics in aquaculture. J. Fish Dis. 25:633-642.

Jernberg, C., Sullivan, A., Edlund, C., Jansson, J.K. (2005) Monitoring of antibiotic-induced alterations in the human intestinal microflora and detection of probiotic strains by use of terminal restriction fragment length polymorphism. Appl. Environ. Microbiol. 71:501-506.

Kanagawa, T. (2003) Bias and artifacts in multitemplate polymerase chain reactions (PCR). J. Biosci. Bioeng. 96:317-323.

Kaplan, C.W., Kitts, C.L. (2003) Variation between observed and true Terminal Restriction Fragment length is dependent on true TRF length and purine content. J. Microbiol. Methods 54:121-125.

Korsnes, K., Nicolaisen, O., Skar, C.K., Nerland, A.H., Bergh, Ø. (2006) Bacteria in the gut of juvenile cod <u>Gadus morhua</u> fed live feed enriched with four different commercial diets. ICES J. Mar. Sci. 63:296-301.

Liu, W.T., Marsh, T.L., Cheng, H., Forney, L.J. (1997) Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. Appl. Environ. Microbiol. 63:4516-4522.

Makridis, P., Fjellheim, A.J., Skjermo, J., Vadstein, O. (2000a) Control of the bacterial flora of Brachionus plicatilis and Artemia franciscana by incubation in bacterial suspensions. Aquaculture 185:207-218.

Makridis, P., Fjellheim, A.J., Skjermo, J., Vadstein, O. (2000b) Colonization of the gut in first feeding turbot by bacterial strains added to the water or bioencapsulated in rotifers. Aquacult. Int. 8:367-380.

Mangor Jensen, A., Adoff, G.R. (1987) Drinking activity of the newly hatched larvae of cod Gadus morhua l. Fish Physiol. Biochem. 3:99-103.

Munro, P.D., Birkbeck, T.H., Barbour, A. (1993) Bacterial flora of rotifers (Brachionus plicatilis): Evidence for a major location on the external surface and methods for reducing the rotifer bacterial load. In: Reinertsen, Dahle, Jørgensen, Tvinnerheim (Eds.) Fish farming technology. Balkema, Rotterdam, pp 93-100.

Munro, P.D., Henderson, R.J., Barbour, A., Birbeck, T.H. (1999) Partial decontamination of rotifers with ultraviolet radiation: the effect of changes in the bacterial load and flora of rotifers on mortalities in start-feeding larval turbot. Aquaculture 170:229-244.

Nair, S., Bharathi, P.A.L., Chandramohan, D. (1994) Culturable heterotrophic bacteria from the euphotic zone of the indian-ocean during the summer monsoon. Oceanol. ACTA 17:63-68.

Oksanen, J., Kindt, R., O`Hara, R.B. (2005) vegan: Community Ecology Package. http://cc.oulu.fi/~jarioksa/.

Olsen, A.I., Olsen, Y., Attramadal, Y., Christie, K., Birkbeck, T.H., Skjermo, J., Vadstein, O. (2000) Effects of short term feeding of microalgae on the bacterial flora associated with juvenile <u>Artemia franciscana</u>. Aquaculture 190:11-25.

Osborn, A.M., Moore, E.R.B., Timmis, K.N. (2000) An evaluation of terminal-restriction fragment length polymorphism (T-RFLP) analysis for the study of microbial community structure and dynamics. Environ. Microbiol. 2:39-50.

Osborne, C.A., Rees, G.N., Bernstein, Y., Janssen, P.H. (2006) New threshold and confidence estimates for terminal restriction fragment length polymorphism analysis of complex bacterial communities. Appl. Environ. Microbiol. 72:1270-1278.

Papakostas, S., Dooms, S., Triantafyllidis, A., Deloof, D., Kappas, I., Dierckens, K., De Wolf, T., Bossier, P., Vadstein, O., Kui, S., Sorgeloos, P., Abatzopoulos, T.J. (2006) Evaluation of DNA methodologies in identifying <u>Brachionus</u> species used in European hatcheries. Aquaculture 255:557-564.

Park, H.G., Puvanendran, V., Kellett, A., Parrish, C.C., Brown, J.A. (2006) Effect of enriched rotifers on growth, survival, and composition of larval Atlantic cod (<u>Gadus morhua</u>). ICES J. Mar. Sci. 63:285-295.

R Development Core Team (2005) R: A language and environment for statistical computing. http://www.R-project.org.

Reitan, K.I., Natvik, C.M., Vadstein, O. (1998) Drinking rate, uptake of bacteria and microalgae in turbot larvae. J. Fish Biol. 53:1145-1154.

Ringø, E., Birkbeck, T.H., Munro, P.D., Vadstein, O., Hjelmeland, K. (1996) The effect of early exposure to <u>Vibrio pelagius</u> on the aerobic bacterial flora of turbot, <u>Scophthalmus maximus</u> (L) larvae. J. Appl. Bacteriol. 81:207-211.

Ringø, E., Vadstein, O. (1998) Colonization of Vibrio pelagius and Aeromonas caviae in early developing turbot (Scophthalmus maximus L.) larvae. J. Appl. Microbiol. 84:227-233.

Salvesen, I., Skjermo, J., Vadstein, O. (1999) Growth of turbot (Scophthalmus maximus L.) during first feeding in relation to the proportion of r/K-strategists in the bacterial community of the rearing water. Aquaculture 175:337-350.

Salvesen, I., Vadstein, O. (1995) Surface disinfection of eggs from marine fish: evaluation of four chemicals. Aquacult. Int. 3:155-171.

Salvesen, I., Vadstein, O. (2000) Evaluation of plate count methods for determination of maximum specific growth rate in mixed microbial communities, and its possible application for diversity assessment. J. Appl. Microbiol. 88:442-448.

Skjermo, J., Bergh, Ø. (2004) High-M alginate immunostimulation of Atlantic halibut (Hippoglossus hippoglossus L.) larvae using Artemia for delivery, increases resistance against vibriosis. Aquaculture 238:107-113.

Skjermo, J., Salvesen, I., Øie, G., Olsen, Y., Vadstein, O. (1997) Microbially matured water: A technique for selection of a non-opportunistic bacterial flora in water that may improve performance of marine larvae. Aquacult. Int. 5:13-28.

Skjermo, J., Størseth, T.R., Hansen, K., Handå, A., Øie, G. (2006) Evaluation of β-(1→3, 1→6)-glucans and High-M alginate used as immunostimulatory dietary supplement during first feeding and weaning of Atlantic cod (*Gadhus morhua* L.). Aquaculture, accepted.

Skjermo, J., Vadstein, O. (1999) Techniques for microbial control in the intensive rearing of marine larvae. Aquaculture 177:333-343.

Suzuki, M.T., Giovannoni, S.J. (1996) Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. Appl. Environ. Microbiol. 62:625-630.

Taoka, Y., Maeda, H., Jo, J.Y., Jeon, M.J., Bai, S.C., Lee, W.J., Yuge, K., Koshio, S. (2006) Growth, stress tolerance and non-specific immune response of Japanese flounder <u>Paralichthys olivaceus</u> to probiotics in a closed recirculating system. Fish. Sci. 72:310-321.

Vaahtovuo, J., Toivanen, P., Eerola, E. (2003) Bacterial composition of murine fecal microflora is indigenous and genetically guided. FEMS Microbiol. Ecol. 44:131-136.

Vadstein, O. (1997) The use of immunostimulation in marine larviculture: possibilities and challenges. Aquaculture 155:401-417.

Verschuere, L., Dhont, J., Sorgeloos, P., Verstraete, W. (1997) Monitoring Biolog patterns and <u>r/K</u>-strategists in the intensive culture of <u>Artemia</u> juveniles. J. Appl. Microbiol. 83:603-612.

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

Vine, N.G., Leukes, W.D., Kaiser, H., Daya, S., Baxter, J., Hecht, T. (2004) Competition for attachment of aquaculture candidate probiotic and pathogenic bacteria on fish intestinal mucus. J. Fish Dis. 27:319-326.

Table 1. Properties of the five probiotic candidates that were added in treatments D and A. The two columns on antagonism indicates how many of the four pathogen and the three dominant bacteria that were inhibited. The line on extracellular enzymeproduction refers to the total score for all 19 extracellular enzymes, maximum score 5 for each of the 19 enzymes with a maximum total of 95. The line on number of enzymes produced refers only to production enzymes with higher score than 2. Expected and observed lengths of the 5´ TRFs for the five probiotic candidates when cut with the restriction enzyme AluI are also shown. Data from Fjellheim et al. in prep. c, except size of TRF.

| | Treatment D | | | Treatment A | |
|---|---|---|---|---|---|
| | ID3-10 | ND2-7 | RD5-30 | RA4-1 | RA7-14 |
| Accession number | **DQ228737** | **DQ228729** | **DQ228725** | **DQ273862** | **DQ273863** |
| Closest match GenBank | Microbacterium sp. **AY730719** | Microbacterium sp. **AY730719** | Vibrio gallicus **AJ440009** | Roseobacter sp. **AY745859** | Pseudolateromonas sp. **AM111084** |
| Selection strategy | Dominant | Dominant | Dominant | Antagonist | Antagonist |
| Antagonism to pathogens | 0 | 0 | 0 | 4 | 3 |
| Antagonism to dominante | 0 | 0 | 0 | 1 | 1 |
| Adhesion to mucus (%) | 5 | 1.5 | 4 | 20 | 2 |
| Growth rate in mucus | 0.039 | 0.033 | 0.075 | No growth | 0.097 |
| Growth rate in marine broth | 0.133 | 0.130 | 0.248 | 0.166 | 0.217 |
| Extracellular enzymeprod. | 47 | 42 | 22 | 22 | 47 |
| Number of enzymes produced | 11 | 9 | 5 | 3 | 9 |
| Fermentative OF medium | - | - | + | - | - |
| Urease activity | + | - | - | + | + |
| Observed TRF | 71 | 71 | 187 | 249 | 239 |
| Expected TRF | 71 | 72 | 187 | 249 | 240 |

Table 2. CFU ($\times 10^3$) in water (ml$^{-1}$), rotifers (ind$^{-1}$) and cod larvae (ind$^{-1}$, n=10). PV(2) indicates the percentage of fast-growing bacterial colonies. TCBS agar is selective for growth of <u>Vibrio</u>. Mean values for the 3 replicate tanks ± SD are given (NA: not analyzed).

| | Treatment | | |
|---|---|---|---|
| Sample | D | A | C |
| CFU water day 0 | 179.8±30.4 | 269.3±44.0 | 102.0±16.7 |
| PV(2)% | 52.3±5.1 | 73.2±6.5 | 49.4±18.1 |
| | | | |
| CFU rotifers day 5 | 7.2±3.9 | 32.2±28.4 | 16.8±5.5 |
| PV(2)% | 17.7±5.3 | 35.8±6.2 | 38.7±30.7 |
| CFU rotifers day 17 | 1.2±0.6 | 0.9±0.3 | 0.8±0.2 |
| PV(2)% | 12.3±2.7 | 10.1±6.1 | 24.7±13.5 |
| | | | |
| CFU larvae day 5 | 35.5±39.7 | 26.4±30.0 | 11.0±12.2 |
| PV(2)% | 35.4±21.0 | 48.7±26.3 | 40.2±15.8 |
| Haemolytic % | NA | 37.9±12.4 | 33.2±26.1 |
| TCBS % | 18.7±21.0 | 45.6±16.5 | 31.0±25.3 |
| CFU larvae day 17 | 95.5±25.0 | 170.1±200.0 | 15.1±3.2 |
| PV(2)% | 13.1±4.8 | 58.9±46.8 | 11.8±11.5 |
| Haemolytic % | 8.3±0.6 | 15.1±22.7 | 13.7±19.1 |
| TCBS % | 11.0±2.1 | 53.1±43.1 | 23.0±30.7 |

Table 3. Percentage of observed TRF±1 for the added probiotic candidates in tank water.

Mean values for the 3 replicate tanks ± SD are given (ND: not detected).

|  | Treatment | Added to dominant treatment | | Added to antagonistic treatment | |
|---|---|---|---|---|---|
|  |  | 71±1 bp (ID3-10/ND2-7) | 187±1 bp (RD5-30) | 239±1 bp (RA7-14) | 249±1 bp (RA4-1) |
| Day 0 | D | 47.0±3.6 | 0.3±0.2 | 2.4±1.1 | 1.2±0.4 |
|  | A | 34.5±3.7 | ND | 13.2±10.7 | 22.8±5.9 |
|  | C | 55.1±14.8 | ND | 2.2±1.0 | 1.3±0.5 |
| Day 5 | D | 3.7±1.1 | 0.2±0.3 | 0.5±0.1 | 2.8±0.7 |
|  | A | 5.2±0.6 | ND | 1.6±2.5 | 3.7±0.7 |
|  | C | 7.5±3.8 | ND | 0.3±0.3 | 2.7±1.2 |
| Day 10 | D | 12.9±5.2 | ND | 2.5±1.8 | 4.1±0.8 |
|  | A | 9.7±1.4 | ND | 1.7±1.2 | 6.9±2.0 |
|  | C | 13.8±4.8 | ND | 2.0±0.5 | 5.4±1.6 |
| Day 17 | D | 6.7±2.8 | ND | 2.3±0.9 | 11.6±5.0 |
|  | A | 12.4±6.9 | ND | 2.6±4.1 | 10.8±1.8 |
|  | C | 22.3±5.3 | ND | 2.8±1.0 | 12.3±1.7 |
| Day 31 | D | 79.2±6.3 | ND | 0.3±0.3 | 0.4±0.5 |
|  | A | 62.4±7.3 | ND | 0.8±1.0 | 1.5±1.7 |
|  | C | 65.8±5.6 | ND | 1.1±1.1 | 0.3±0.6 |

Table 4. Percentage of observed TRF±1 of the added probiotic candidates in rotifers after 30 min in bacterial suspensions and 5 min wash with brackish water.

| | Treatment | Added to dominant treatment | | Added to antagonistic treatment | |
|---|---|---|---|---|---|
| | | 71±1 bp (ID3-10/ND2-7) | 187±1 bp (RD5-30) | 239±1 bp (RA7-14) | 249±1 bp (RA4-1) |
| Day 4 | D | 18.5 | 5.4 | 38.1 | 6.5 |
| | A | 8.0 | 0.6 | 27.9 | 31.4 |
| | C | 9.7 | 0.0 | 34.6 | 12.0 |
| Day 11 | D | 2.2 | 5.1 | 2.5 | 14.7 |
| | A | 1.7 | 1.4 | 36.4 | 29.5 |
| | C | 0.9 | 0.0 | 2.4 | 10.8 |

Table 5. Percentage of observed TRF±1 of the added probiotic candidates in rotifers from the tank water. Mean values for the 3 replicate tanks ± SD are given (ND: not detected).

| | Treatment | Added to dominant treatment | | Added to antagonistic treatment | |
| | | 71±1 bp (ID3-10/ND2-7) | 187±1 bp (RD5-30) | 239±1 bp (RA7-14) | 249±1 bp (RA4-1) |
|---|---|---|---|---|---|
| Day 5 | D | 48.0±9.1 | 4.5±5.5 | 14.0±11.9 | 3.5±1.9 |
| | A | 20.7±11.7 | ND | 19.1±6.6 | 6.2±1.8 |
| | C | 27.5±7.0 | ND | 13.7±10.7 | 4.4±0.2 |
| Day 10 | D | 23.7±26.6 | ND | 5.1±3.5 | 2.9±0.6 |
| | A | 34.2±5.7 | ND | 8.2±0.4 | 3.4±1.2 |
| | C | 33.3±13.8 | ND | 5.6±5.0 | 2.6±2.5 |
| Day 17 | D | 34.2±5.3 | ND | 7.8±2.6 | 7.7±5.0 |
| | A | 34.2±30.0 | ND | 4.3±0.7 | 6.4±8.2 |
| | C | 27.3±15.0 | ND | 5.8±5.8 | 5.7±4.2 |

Table 6. Percentage of observed TRF±1 of the added probiotic candidates in cod larvae. Mean values for the 3 replicate tanks ± SD are given (ND: not detected).

| | Treatment | Added to dominant treatment | | Added to antagonistic treatment | |
|---|---|---|---|---|---|
| | | 71±1 bp (ID3-10/ND2-7) | 187±1 bp (RD5-30) | 239±1 bp (RA7-14) | 249±1 bp (RA4-1) |
| Day 5 | D | 17.8±11.2 | ND | 35.5±21.1 | 3.6±2.7 |
| | A | 17.7±19.1 | ND | 26.4±26.0 | 2.7±4.0 |
| | C | 38.8±22.3 | ND | 32.6±26.2 | 3.9±5.4 |
| Day 10 | D | 5.9±7.7 | ND | 69.3±8.0 | 0.3±0.5 |
| | A | 26.7±36.9 | ND | 29.0±41.9 | 0.5±0.9 |
| | C | 4.4±0.2 | ND | 34.6±31.9 | 3.4±4.5 |
| Day 17 | D | 13.2±6.5 | 1.0±1.0 | 37.9±18.9 | 3.5±2.3 |
| | A | 23.6±12.1 | ND | 32.1±27.6 | 3.6±1.0 |
| | C | 5.9±8.3 | ND | 32.6±12.2 | 0.8±1.2 |
| Day 31 | D | 47.6±12.9 | ND | 21.7±18.8 | 1.9±2.3 |
| | A | 39.0±35.8 | ND | 0.9±1.2 | ND |
| | C | 51.3±23.5 | ND | 17.1±14.9 | 2.0±2.7 |

Table 7. Larval dry weight ($\mu$g larvae$^{-1}$, n=12) and percentage specific growth rate (SGR) day$^{-1}$. Mean values for the 3 replicate tanks $\pm$ SD are given.

| | Treatment D | | | | Treatment A | | | | Control | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Tank 1 | Tank 2 | Tank 3 | Sum D | Tank 1 | Tank 2 | Tank 3 | Sum A | Tank 1 | Tank 2 | Tank 3 | Sum C |
| DW day 5 | 105±20 | 116±46 | 107±52 | 109±41 | 118±51 | 109±90 | 83±17 | 103±61 | 69±12 | 63±12 | 105±34 | 79±29 |
| DW day 10 | 148±60 | 97±17 | 208±72 | 151±70 | 120±29 | 173±48 | 129±16 | 141±40 | 112±18 | 175±38 | 111±36 | 133±44 |
| DW day 17 | 548±254 | 657±216 | 584±195 | 596±223 | 517±153 | 500±168 | 497±181 | 505±164 | 701±305 | 625±205 | 632±257 | 653±255 |
| DW day 31 | 947±320 | 957±481 | 1161±327 | 1011±357 | 1130±277 | 939±325 | 821±178 | 966±287 | 987±307 | 1155±452 | 865±334 | 969±372 |
| %SGR day 5-10 | 7.2 | -3.4 | 14.3 | 6.8 | 0.3 | 9.7 | 9.4 | 6.4 | 10.3 | 22.9 | 1.1 | 11.0 |
| %SGR day 10-17 | 20.6 | 31.4 | 15.9 | 21.7 | 23.4 | 16.3 | 21.2 | 20.0 | 30.0 | 20.0 | 28.3 | 25.6 |
| %SGR day 17-31 | 4.0 | 2.7 | 5.0 | 3.9 | 5.7 | 4.6 | 3.7 | 4.7 | 2.5 | 4.5 | 2.3 | 3.1 |

**Figure legends:**

Figure 1. Distribution of different TRFs in four individual cod larvae from each of the three tanks in treatment D, A and C on day 17 after hatching. To the right the average in larvae from each treatment is shown. TRF 71±1 corresponds to ID3-10 and ND2-7 added in treatment D, TRF 187±1 corresponds to RD5-30 added in treatment D, TRF 239±1 corresponds to RA7-14 added in treatment A, and TRF 249±1 corresponds to RA4-1 added in treatment A.

Figure 2. Survival during weaning for larvae in the treated tanks and the control, error bars show standard deviation. (●) Treatment D, (○) treatment A, (▼) control.
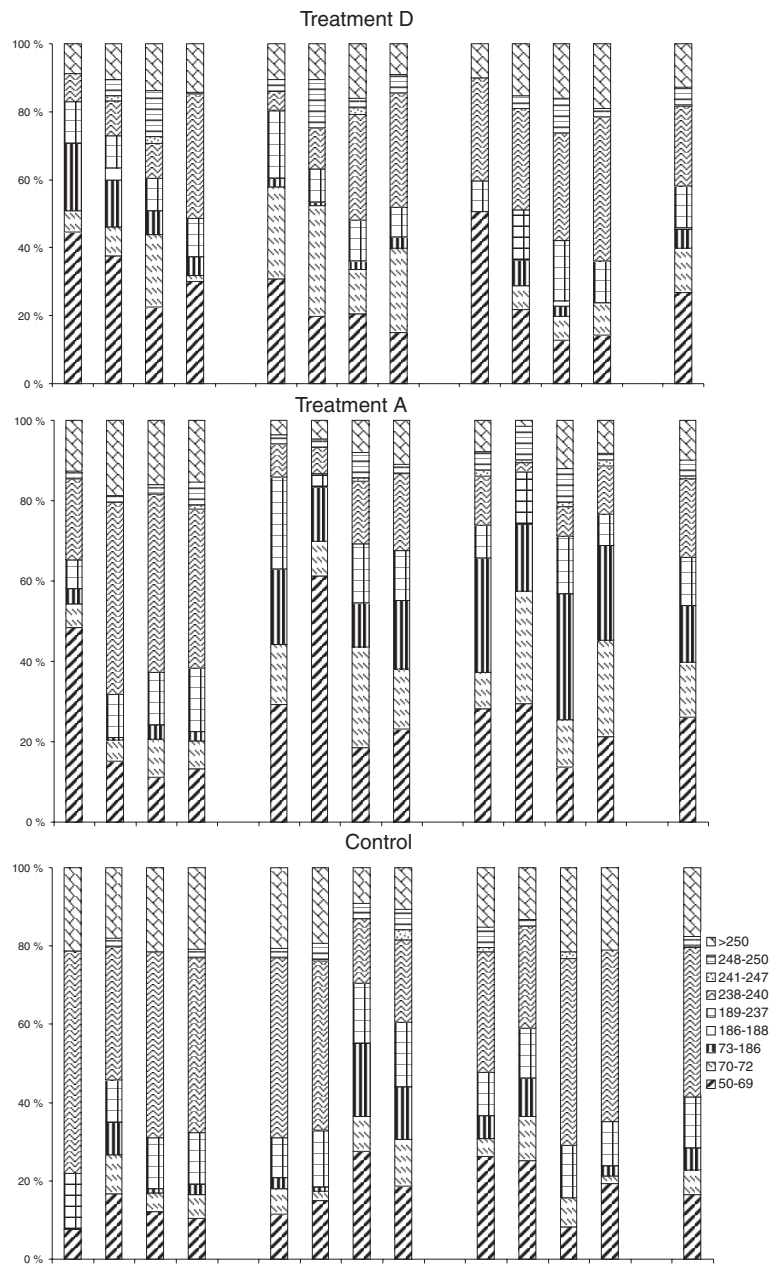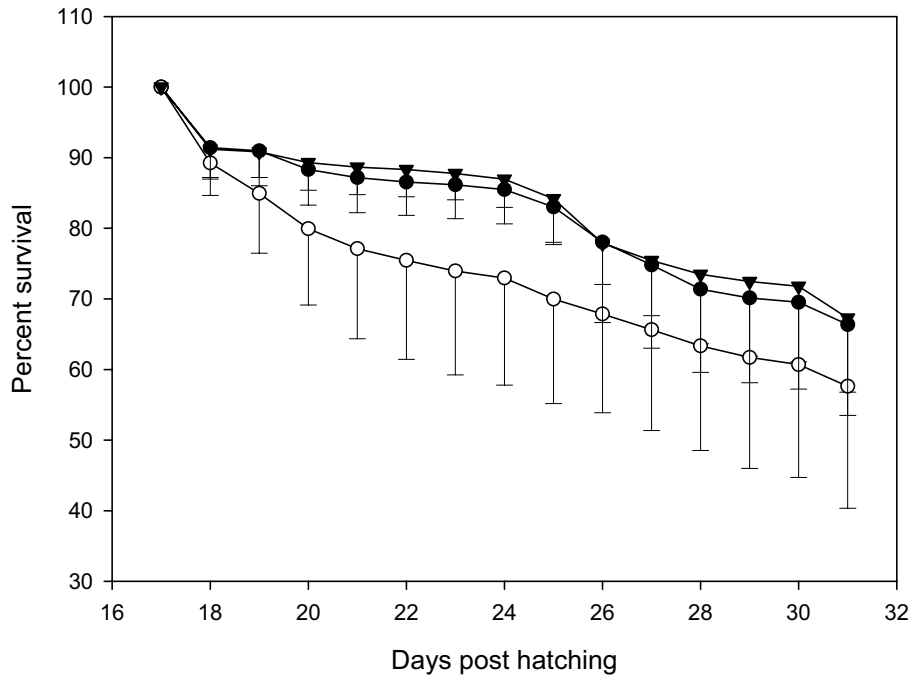
Figure 1

**Figure 2**

# Doctoral theses in Biology
## Norwegian University of Science and Technology
## Department of Biology

| Year | Name | Degree | Title |
|------|------|--------|-------|
| 1974 | Tor-Henning Iversen | Dr. philos Botany | The roles of statholiths, auxin transport, and auxin metabolism in root gravitropism |
| 1978 | Tore Slagsvold | Dr. philos. Zoology | Breeding events of birds in relation to spring temperature and environmental phenology. |
| 1978 | Egil Sakshaug | Dr.philos Botany | "The influence of environmental factors on the chemical composition of cultivated and natural populations of marine phytoplankton" |
| 1980 | Arnfinn Langeland | Dr. philos. Zoology | Interaction between fish and zooplankton populations and their effects on the material utilization in a freshwater lake. |
| 1980 | Helge Reinertsen | Dr. philos Botany | The effect of lake fertilization on the dynamics and stability of a limnetic ecosystem with special reference to the phytoplankton |
| 1982 | Gunn Mari Olsen | Dr. scient Botany | Gravitropism in roots of *Pisum sativum* and *Arabidopsis thaliana* |
| 1982 | Dag Dolmen | Dr. philos. Zoology | Life aspects of two sympartic species of newts (*Triturus, Amphibia*) in Norway, with special emphasis on their ecological niche segregation. |
| 1984 | Eivin Røskaft | Dr. philos. Zoology | Sociobiological studies of the rook *Corvus frugilegus.* |
| 1984 | Anne Margrethe Cameron | Dr. scient Botany | Effects of alcohol inhalation on levels of circulating testosterone, follicle stimulating hormone and luteinzing hormone in male mature rats |
| 1984 | Asbjørn Magne Nilsen | Dr. scient Botany | Alveolar macrophages from expectorates – Biological monitoring of workers exosed to occupational air pollution. An evaluation of the AM-test |
| 1985 | Jarle Mork | Dr. philos. Zoology | Biochemical genetic studies in fish. |
| 1985 | John Solem | Dr. philos. Zoology | Taxonomy, distribution and ecology of caddisflies (*Trichoptera*) in the Dovrefjell mountains. |
| 1985 | Randi E. Reinertsen | Dr. philos. Zoology | Energy strategies in the cold: Metabolic and thermoregulatory adaptations in small northern birds. |
| 1986 | Bernt-Erik Sæther | Dr. philos. Zoology | Ecological and evolutionary basis for variation in reproductive traits of some vertebrates: A comparative approach. |
| 1986 | Torleif Holthe | Dr. philos. Zoology | Evolution, systematics, nomenclature, and zoogeography in the polychaete orders *Oweniimorpha* and *Terebellomorpha*, with special reference to the Arctic and Scandinavian fauna. |
| 1987 | Helene Lampe | Dr. scient. Zoology | The function of bird song in mate attraction and territorial defence, and the importance of song repertoires. |
| 1987 | Olav Hogstad | Dr. philos. Zoology | Winter survival strategies of the Willow tit *Parus montanus.* |

| | | |
|---|---|---|
| 1987 Jarle Inge Holten | Dr. philos Bothany | Autecological investigations along a coust-inland transect at Nord-Møre, Central Norway |
| 1987 Rita Kumar | Dr. scient Botany | Somaclonal variation in plants regenerated from cell cultures of *Nicotiana sanderae* and *Chrysanthemum morifolium* |
| 1987 Bjørn Åge Tømmerås | Dr. scient. Zoology | Olfaction in bark beetle communities: Interspecific interactions in regulation of colonization density, predator - prey relationship and host attraction. |
| 1988 Hans Christian Pedersen | Dr. philos. Zoology | Reproductive behaviour in willow ptarmigan with special emphasis on territoriality and parental care. |
| 1988 Tor G. Heggberget | Dr. philos. Zoology | Reproduction in Atlantic Salmon (*Salmo salar*): Aspects of spawning, incubation, early life history and population structure. |
| 1988 Marianne V. Nielsen | Dr. scient. Zoology | The effects of selected environmental factors on carbon allocation/growth of larval and juvenile mussels (*Mytilus edulis*). |
| 1988 Ole Kristian Berg | Dr. scient. Zoology | The formation of landlocked Atlantic salmon (*Salmo salar* L.). |
| 1989 John W. Jensen | Dr. philos. Zoology | Crustacean plankton and fish during the first decade of the manmade Nesjø reservoir, with special emphasis on the effects of gill nets and salmonid growth. |
| 1989 Helga J. Vivås | Dr. scient. Zoology | Theoretical models of activity pattern and optimal foraging: Predictions for the Moose *Alces alces*. |
| 1989 Reidar Andersen | Dr. scient. Zoology | Interactions between a generalist herbivore, the moose *Alces alces*, and its winter food resources: a study of behavioural variation. |
| 1989 Kurt Ingar Draget | Dr. scient Botany | Alginate gel media for plant tissue culture, |
| 1990 Bengt Finstad | Dr. scient. Zoology | Osmotic and ionic regulation in Atlantic salmon, rainbow trout and Arctic charr: Effect of temperature, salinity and season. |
| 1990 Hege Johannesen | Dr. scient. Zoology | Respiration and temperature regulation in birds with special emphasis on the oxygen extraction by the lung. |
| 1990 Åse Krøkje | Dr. scient Botany | The mutagenic load from air pollution at two work-places with PAH-exposure measured with Ames Salmonella/microsome test |
| 1990 Arne Johan Jensen | Dr. philos. Zoology | Effects of water temperature on early life history, juvenile growth and prespawning migrations of Atlantic salmion (*Salmo salar*) and brown trout (*Salmo trutta*): A summary of studies in Norwegian streams. |
| 1990 Tor Jørgen Almaas | Dr. scient. Zoology | Pheromone reception in moths: Response characteristics of olfactory receptor neurons to intra- and interspecific chemical cues. |
| 1990 Magne Husby | Dr. scient. Zoology | Breeding strategies in birds: Experiments with the Magpie *Pica pica*. |
| 1991 Tor Kvam | Dr. scient. Zoology | Population biology of the European lynx (*Lynx lynx*) in Norway. |
| 1991 Jan Henning L'Abêe Lund | Dr. philos. Zoology | Reproductive biology in freshwater fish, brown trout *Salmo trutta* and roach *Rutilus rutilus* in particular. |
| 1991 Asbjørn Moen | Dr. philos Botany | The plant cover of the boreal uplands of Central Norway. I. Vegetation ecology of Sølendet nature reserve; haymaking fens and birch woodlands |
| 1991 Else Marie Løbersli | Dr. scient Botany | Soil acidification and metal uptake in plants |

| | | | |
|---|---|---|---|
| 1991 | Trond Nordtug | Dr. scient. Zoology | Reflctometric studies of photomechanical adaptation in superposition eyes of arthropods. |
| 1991 | Thyra Solem | Dr. scient Botany | Age, origin and development of blanket mires in Central Norway |
| 1991 | Odd Terje Sandlund | Dr. philos. Zoology | The dynamics of habitat use in the salmonid genera *Coregonus* and *Salvelinus*: Ontogenic niche shifts and polymorphism. |
| 1991 | Nina Jonsson | Dr. philos. | Aspects of migration and spawning in salmonids. |
| 1991 | Atle Bones | Dr. scient Botany | Compartmentation and molecular properties of thioglucoside glucohydrolase (myrosinase) |
| 1992 | Torgrim Breiehagen | Dr. scient. Zoology | Mating behaviour and evolutionary aspects of the breeding system of two bird species: the Temminck's stint and the Pied flycatcher. |
| 1992 | Anne Kjersti Bakken | Dr. scient Botany | The influence of photoperiod on nitrate assimilation and nitrogen status in timothy (*Phleum pratense* L.) |
| 1992 | Tycho Anker-Nilssen | Dr. scient. Zoology | Food supply as a determinant of reproduction and population development in Norwegian Puffins *Fratercula arctica* |
| 1992 | Bjørn Munro Jenssen | Dr. philos. Zoology | Thermoregulation in aquatic birds in air and water: With special emphasis on the effects of crude oil, chemically treated oil and cleaning on the thermal balance of ducks. |
| 1992 | Arne Vollan Aarset | Dr. philos. Zoology | The ecophysiology of under-ice fauna: Osmotic regulation, low temperature tolerance and metabolism in polar crustaceans. |
| 1993 | Geir Slupphaug | Dr. scient Botany | Regulation and expression of uracil-DNA glycosylase and $O^6$-methylguanine-DNA methyltransferase in mammalian cells |
| 1993 | Tor Fredrik Næsje | Dr. scient. Zoology | Habitat shifts in coregonids. |
| 1993 | Yngvar Asbjørn Olsen | Dr. scient. Zoology | Cortisol dynamics in Atlantic salmon, *Salmo salar* L.: Basal and stressor-induced variations in plasma levels ans some secondary effects. |
| 1993 | Bård Pedersen | Dr. scient Botany | Theoretical studies of life history evolution in modular and clonal organisms |
| 1993 | Ole Petter Thangstad | Dr. scient Botany | Molecular studies of myrosinase in Brassicaceae |
| 1993 | Thrine L. M. Heggberget | Dr. scient. Zoology | Reproductive strategy and feeding ecology of the Eurasian otter *Lutra lutra*. |
| 1993 | Kjetil Bevanger | Dr. scient. Zoology | Avian interactions with utility structures, a biological approach. |
| 1993 | Kåre Haugan | Dr. scient Bothany | Mutations in the replication control gene trfA of the broad host-range plasmid RK2 |
| 1994 | Peder Fiske | Dr. scient. Zoology | Sexual selection in the lekking great snipe (*Gallinago media*): Male mating success and female behaviour at the lek. |
| 1994 | Kjell Inge Reitan | Dr. scient Botany | Nutritional effects of algae in first-feeding of marine fish larvae |
| 1994 | Nils Røv | Dr. scient. Zoology | Breeding distribution, population status and regulation of breeding numbers in the northeast-Atlantic Great Cormorant *Phalacrocorax carbo carbo*. |
| 1994 | Annette-Susanne Hoepfner | Dr. scient Botany | Tissue culture techniques in propagation and breeding of Red Raspberry (*Rubus idaeus* L.) |
| 1994 | Inga Elise Bruteig | Dr. scient Bothany | Distribution, ecology and biomonitoring studies of epiphytic lichens on conifers |

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|---|---|---|
| 1994 Geir Johnsen | Dr. scient Botany | Light harvesting and utilization in marine phytoplankton: Species-specific and photoadaptive responses |
| 1994 Morten Bakken | Dr. scient. Zoology | Infanticidal behaviour and reproductive performance in relation to competition capacity among farmed silver fox vixens, *Vulpes vulpes*. |
| 1994 Arne Moksnes | Dr. philos. Zoology | Host adaptations towards brood parasitism by the Cockoo. |
| 1994 Solveig Bakken | Dr. scient Bothany | Growth and nitrogen status in the moss *Dicranum majus* Sm. as influenced by nitrogen supply |
| 1995 Olav Vadstein | Dr. philos Botany | The role of heterotrophic planktonic bacteria in the cycling of phosphorus in lakes: Phosphorus requirement, competitive ability and food web interactions. |
| 1995 Hanne Christensen | Dr. scient. Zoology | Determinants of Otter *Lutra lutra* distribution in Norway: Effects of harvest, polychlorinated biphenyls (PCBs), human population density and competition with mink *Mustela vision*. |
| 1995 Svein Håkon Lorentsen | Dr. scient. Zoology | Reproductive effort in the Antarctic Petrel *Thalassoica antarctica*; the effect of parental body size and condition. |
| 1995 Chris Jørgen Jensen | Dr. scient. Zoology | The surface electromyographic (EMG) amplitude as an estimate of upper trapezius muscle activity |
| 1995 Martha Kold Bakkevig | Dr. scient. Zoology | The impact of clothing textiles and construction in a clothing system on thermoregulatory responses, sweat accumulation and heat transport. |
| 1995 Vidar Moen | Dr. scient. Zoology | Distribution patterns and adaptations to light in newly introduced populations of *Mysis relicta* and constraints on Cladoceran and Char populations. |
| 1995 Hans Haavardsholm Blom | Dr. philos Bothany | A revision of the *Schistidium apocarpum* complex in Norway and Sweden. |
| 1996 Jorun Skjærmo | Dr. scient Botany | Microbial ecology of early stages of cultivated marine fish; inpact fish-bacterial interactions on growth and survival of larvae. |
| 1996 Ola Ugedal | Dr. scient. Zoology | Radiocesium turnover in freshwater fishes |
| 1996 Ingibjørg Einarsdottir | Dr. scient. Zoology | Production of Atlantic salmon (*Salmo salar*) and Arctic charr (*Salvelinus alpinus*): A study of some physiological and immunological responses to rearing routines. |
| 1996 Christina M. S. Pereira | Dr. scient. Zoology | Glucose metabolism in salmonids: Dietary effects and hormonal regulation. |
| 1996 Jan Fredrik Børseth | Dr. scient. Zoology | The sodium energy gradients in muscle cells of *Mytilus edulis* and the effects of organic xenobiotics. |
| 1996 Gunnar Henriksen | Dr. scient. Zoology | Status of Grey seal *Halichoerus grypus* and Harbour seal *Phoca vitulina* in the Barents sea region. |
| 1997 Gunvor Øie | Dr. scient Bothany | Eevaluation of rotifer *Brachionus plicatilis* quality in early first feeding of turbot *Scophtalmus maximus* L. larvae. |
| 1997 Håkon Holien | Dr. scient Botany | Studies of lichens in spurce forest of Central Norway. Diversity, old growth species and the relationship to site and stand parameters. |
| 1997 Ole Reitan | Dr. scient. Zoology | Responses of birds to habitat disturbance due to damming. |
| 1997 Jon Arne Grøttum | Dr. scient. Zoology | Physiological effects of reduced water quality on fish in aquaculture. |

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|---|---|---|
| 1997 Per Gustav Thingstad | Dr. scient. Zoology | Birds as indicators for studying natural and human-induced variations in the environment, with special emphasis on the suitability of the Pied Flycatcher. |
| 1997 Torgeir Nygård | Dr. scient. Zoology | Temporal and spatial trends of pollutants in birds in Norway: Birds of prey and Willow Grouse used as Biomonitors. |
| 1997 Signe Nybø | Dr. scient. Zoology | Impacts of long-range transported air pollution on birds with particular reference to the dipper *Cinclus cinclus* in southern Norway. |
| 1997 Atle Wibe | Dr. scient. Zoology | Identification of conifer volatiles detected by receptor neurons in the pine weevil (*Hylobius abietis*), analysed by gas chromatography linked to electrophysiology and to mass spectrometry. |
| 1997 Rolv Lundheim | Dr. scient. Zoology | Adaptive and incidental biological ice nucleators. |
| 1997 Arild Magne Landa | Dr. scient. Zoology | Wolverines in Scandinavia: ecology, sheep depredation and conservation. |
| 1997 Kåre Magne Nielsen | Dr. scient Botany | An evolution of possible horizontal gene transfer from plants to sail bacteria by studies of natural transformation in *Acinetobacter calcoacetius*. |
| 1997 Jarle Tufto | Dr. scient. Zoology | Gene flow and genetic drift in geographically structured populations: Ecological, population genetic, and statistical models |
| 1997 Trygve Hesthagen | Dr. philos. Zoology | Population responces of Arctic charr (*Salvelinus alpinus* (L.)) and brown trout (*Salmo trutta* L.) to acidification in Norwegian inland waters |
| 1997 Trygve Sigholt | Dr. philos. Zoology | Control of Parr-smolt transformation and seawater tolerance in farmed Atlantic Salmon (*Salmo salar*) Effects of photoperiod, temperature, gradual seawater acclimation, NaCl and betaine in the diet |
| 1997 Jan Østnes | Dr. scient. Zoology | Cold sensation in adult and neonate birds |
| 1998 Seethaledsumy Visvalingam | Dr. scient Botany | Influence of environmental factors on myrosinases and myrosinase-binding proteins. |
| 1998 Thor Harald Ringsby | Dr. scient. Zoology | Variation in space and time: The biology of a House sparrow metapopulation |
| 1998 Erling Johan Solberg | Dr. scient. Zoology | Variation in population dynamics and life history in a Norwegian moose (*Alces alces*) population: consequences of harvesting in a variable environment |
| 1998 Sigurd Mjøen Saastad | Dr. scient Botany | Species delimitation and phylogenetic relationships between the Sphagnum recurvum complex (Bryophyta): genetic variation and phenotypic plasticity. |
| 1998 Bjarte Mortensen | Dr. scient Botany | Metabolism of volatile organic chemicals (VOCs) in a head liver S9 vial equilibration system in vitro. |
| 1998 Gunnar Austrheim | Dr. scient Botany | Plant biodiversity and land use in subalpine grasslands. – A conservtaion biological approach. |
| 1998 Bente Gunnveig Berg | Dr. scient. Zoology | Encoding of pheromone information in two related moth species |
| 1999 Kristian Overskaug | Dr. scient. Zoology | Behavioural and morphological characteristics in Northern Tawny Owls *Strix aluco*: An intra- and interspecific comparative approach |
| 1999 Hans Kristen Stenøien | Dr. scient Bothany | Genetic studies of evolutionary processes in various populations of nonvascular plants (mosses, liverworts and hornworts) |

| Year | Name | Degree/Field | Title |
|---|---|---|---|
| 1999 | Trond Arnesen | Dr. scient Botany | Vegetation dynamics following trampling and burning in the outlying haylands at Sølendet, Central Norway. |
| 1999 | Ingvar Stenberg | Dr. scient. Zoology | Habitat selection, reproduction and survival in the White-backed Woodpecker *Dendrocopos leucotos* |
| 1999 | Stein Olle Johansen | Dr. scient Botany | A study of driftwood dispersal to the Nordic Seas by dendrochronology and wood anatomical analysis. |
| 1999 | Trina Falck Galloway | Dr. scient. Zoology | Muscle development and growth in early life stages of the Atlantic cod (*Gadus morhua* L.) and Halibut (*Hippoglossus hippoglossus* L.) |
| 1999 | Torbjørn Forseth | Dr. scient. Zoology | Bioenergetics in ecological and life history studies of fishes. |
| 1999 | Marianne Giæver | Dr. scient. Zoology | Population genetic studies in three gadoid species: blue whiting (*Micromisistius poutassou*), haddock (*Melanogrammus aeglefinus*) and cod (*Gradus morhua*) in the North-East Atlantic |
| 1999 | Hans Martin Hanslin | Dr. scient Botany | The impact of environmental conditions of density dependent performance in the boreal forest bryophytes *Dicranum majus*, *Hylocomium splendens*, *Plagiochila asplenigides*, *Ptilium crista-castrensis* and *Rhytidiadelphus lokeus*. |
| 1999 | Ingrid Bysveen Mjølnerød | Dr. scient. Zoology | Aspects of population genetics, behaviour and performance of wild and farmed Atlantic salmon (*Salmo salar*) revealed by molecular genetic techniques |
| 1999 | Else Berit Skagen | Dr. scient Botany | The early regeneration process in protoplasts from *Brassica napus* hypocotyls cultivated under various g-forces |
| 1999 | Stein-Are Sæther | Dr. philos. Zoology | Mate choice, competition for mates, and conflicts of interest in the Lekking Great Snipe |
| 1999 | Katrine Wangen Rustad | Dr. scient. Zoology | Modulation of glutamatergic neurotransmission related to cognitive dysfunctions and Alzheimer's disease |
| 1999 | Per Terje Smiseth | Dr. scient. Zoology | Social evolution in monogamous families: mate choice and conflicts over parental care in the Bluethroat (*Luscinia s. svecica*) |
| 1999 | Gunnbjørn Bremset | Dr. scient. Zoology | Young Atlantic salmon (*Salmo salar* L.) and Brown trout (*Salmo trutta* L.) inhabiting the deep pool habitat, with special reference to their habitat use, habitat preferences and competitive interactions |
| 1999 | Frode Ødegaard | Dr. scient. Zoology | Host spesificity as parameter in estimates of arhrophod species richness |
| 1999 | Sonja Andersen | Dr. scient Bothany | Expressional and functional analyses of human, secretory phospholipase A2 |
| 2000 | Ingrid Salvesen, I | Dr. scient Botany | Microbial ecology in early stages of marine fish: Development and evaluation of methods for microbial management in intensive larviculture |
| 2000 | Ingar Jostein Øien | Dr. scient. Zoology | The Cuckoo (*Cuculus canorus*) and its host: adaptions and counteradaptions in a coevolutionary arms race |
| 2000 | Pavlos Makridis | Dr. scient Botany | Methods for the microbial econtrol of live food used for the rearing of marine fish larvae |
| 2000 | Sigbjørn Stokke | Dr. scient. Zoology | Sexual segregation in the African elephant (*Loxodonta africana*) |
| 2000 | Odd A. Gulseth | Dr. philos. Zoology | Seawater tolerance, migratory behaviour and growth of Charr, (*Salvelinus alpinus*), with emphasis on the high Arctic Dieset charr on Spitsbergen, Svalbard |

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|---|---|---|
| 2000 Pål A. Olsvik | Dr. scient. Zoology | Biochemical impacts of Cd, Cu and Zn on brown trout (*Salmo trutta*) in two mining-contaminated rivers in Central Norway |
| 2000 Sigurd Einum | Dr. scient. Zoology | Maternal effects in fish: Implications for the evolution of breeding time and egg size |
| 2001 Jan Ove Evjemo | Dr. scient. Zoology | Production and nutritional adaptation of the brine shrimp *Artemia* sp. as live food organism for larvae of marine cold water fish species |
| 2001 Olga Hilmo | Dr. scient Botany | Lichen response to environmental changes in the managed boreal forset systems |
| 2001 Ingebrigt Uglem | Dr. scient. Zoology | Male dimorphism and reproductive biology in corkwing wrasse (*Symphodus melops* L.) |
| 2001 Bård Gunnar Stokke | Dr. scient. Zoology | Coevolutionary adaptations in avian brood parasites and their hosts |
| 2002 Ronny Aanes | Dr. scient | Spatio-temporal dynamics in Svalbard reindeer (*Rangifer tarandus platyrhynchus*) |
| 2002 Mariann Sandsund | Dr. scient. Zoology | Exercise- and cold-induced asthma. Respiratory and thermoregulatory responses |
| 2002 Dag-Inge Øien | Dr. scient Botany | Dynamics of plant communities and populations in boreal vegetation influenced by scything at Sølendet, Central Norway |
| 2002 Frank Rosell | Dr. scient. Zoology | The function of scent marking in beaver (*Castor fiber*) |
| 2002 Janne Østvang | Dr. scient Botany | The Role and Regulation of Phospholipase $A_2$ in Monocytes During Atherosclerosis Development |
| 2002 Terje Thun | Dr. philos Biology | Dendrochronological constructions of Norwegian conifer chronologies providing dating of historical material |
| 2002 Birgit Hafjeld Borgen | Dr. scient Biology | Functional analysis of plant idioblasts (Myrosin cells) and their role in defense, development and growth |
| 2002 Bård Øyvind Solberg | Dr. scient Biology | Effects of climatic change on the growth of dominating tree species along major environmental gradients |
| 2002 Per Winge | Dr. scient Biology | The evolution of small GTP binding proteins in cellular organisms. Studies of RAC GTPases in *Arabidopsis thaliana* and |
| 2002 Henrik Jensen | Dr. scient Biology | Causes and consequenses of individual variation in fitness-related traits in house sparrows |
| 2003 Jens Rohloff | Dr. philos Biology | Cultivation of herbs and medicinal plants in Norway – Essential oil production and quality control |
| 2003 Åsa Maria O. Espmark Wibe | Dr. scient Biology | Behavioural effects of environmental pollution in threespine stickleback *Gasterosteus aculeatur* L. |
| 2003 Dagmar Hagen | Dr. scient Biology | Assisted recovery of disturbed arctic and alpine vegetation – an integrated approach |
| 2003 Bjørn Dahle | Dr. scient Biology | Reproductive strategies in Scandinavian brown bears |
| 2003 Cyril Lebogang Taolo | Dr. scient Biology | Population ecology, seasonal movement and habitat use of the African buffalo (*Syncerus caffer*) in Chobe National Park, Botswana |
| 2003 Marit Stranden | Dr.scient Biology | Olfactory receptor neurones specified for the same odorants in three related Heliothine species (*Helicoverpa armigera, Helicoverpa assulta* and *Heliothis virescens*) |
| 2003 Kristian Hassel | Dr.scient Biology | Life history characteristics and genetic variation in an expanding species, *Pogonatum dentatum* |

| Year | Name | Degree | Title |
|---|---|---|---|
| 2003 | David Alexander Rae | Dr.scient Biology | Plant- and invertebrate-community responses to species interaction and microclimatic gradients in alpine and Artic environments |
| 2003 | Åsa A Borg | Dr.scient Biology | Sex roles and reproductive behaviour in gobies and guppies: a female perspective |
| 2003 | Eldar Åsgard Bendiksen | Dr.scient Biology | Environmental effects on lipid nutrition of farmed Atlantic salmon (*Salmo Salar* L.) parr and smolt |
| 2004 | Torkild Bakken | Dr.scient Biology | A revision of Nereidinae (Polychaeta, Nereididae) |
| 2004 | Ingar Pareliussen | Dr.scient Biology | Natural and Experimental Tree Establishment in a Fragmented Forest, Ambohitantely Forest Reserve, Madagascar |
| 2004 | Tore Brembu | Dr.scient Biology | Genetic, molecular and functional studies of RAC GTPases and the WAVE-like regulatory protein complex in *Arabidopsis thaliana* |
| 2004 | Liv S. Nilsen | Dr.scient Biology | Coastal heath vegetation on central Norway; recent past, present state and future possibilities |
| 2004 | Hanne T. Skiri | Dr.scient Biology | Olfactory coding and olfactory learning of plant odours in heliothine moths. An anatomical, physiological and behavioural study of three related species (*Heliothis virescens, Helicoverpa armigera* and *Helicoverpa assulta*). |
| 2004 | Lene Østby | Dr.scient Biology | Cytochrome P4501A (CYP1A) induction and DNA adducts as biomarkers for organic pollution in the natural environment |
| 2004 | Emmanuel J. Gerreta | Dr. philos Biology | The Importance of Water Quality and Quantity in the Tropical Ecosystems, Tanzania |
| 2004 | Linda Dalen | Dr.scient Biology | Dynamics of Mountain Birch Treelines in the Scandes Mountain Chain, and Effects of Climate Warming |
| 2004 | Lisbeth Mehli | Dr.scient Biology | Polygalacturonase-inhibiting protein (PGIP) in cultivated strawberry (*Fragaria* x *ananassa*): characterisation and induction of the gene following fruit infection by *Botrytis cinerea* |
| 2004 | Børge Moe | Dr.scient Biology | Energy-Allocation in Avian Nestlings Facing Short-Term Food Shortage |
| 2005 | Matilde Skogen Chauton | Dr.scient Biology | Metabolic profiling and species discrimination from High-Resolution Magic Angle Spinning NMR analysis of whole-cell samples |
| 2005 | Sten Karlsson | Dr.scient Biology | Dynamics of Genetic Polymorphisms |
| 2005 | Terje Bongard | Dr.scient Biology | Life History strategies, mate choice, and parental investment among Norwegians over a 300-year period |
| 2005 | Tonette Røstelien | PhD Biology | Functional characterisation of olfactory receptor neurone types in heliothine moths |
| 2005 | Erlend Kristiansen | Dr.scient Biology | Studies on antifreeze proteins |
| 2005 | Eugen G. Sørmo | Dr.scient Biology | Organochlorine pollutants in grey seal (*Halichoerus grypus*) pups and their impact on plasma thyrid hormone and vitamin A concentrations. |
| 2005 | Christian Westad | Dr.scient Biology | Motor control of the upper trapezius |

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|---|---|---|---|
| 2005 | Lasse Mork Olsen | PhD Biology | Interactions between marine osmo- and phagotrophs in different physicochemical environments |
| 2005 | Åslaug Viken | PhD Biology | Implications of mate choice for the management of small populations |
| 2005 | Ariaya Hymete Sahle Dingle | PhD Biology | Investigation of the biological activities and chemical constituents of selected *Echinops* spp. growing in Ethiopia |
| 2005 | Ander Gravbrøt Finstad | PhD Biology | Salmonid fishes in a changing climate: The winter challenge |
| 2005 | Shimane Washington Makabu | PhD Biology | Interactions between woody plants, elephants and other browsers in the Chobe Riverfront, Botswana |
| 2005 | Kjartan Østbye | Dr.scient Biology | The European whitefish *Coregonus lavaretus* (L.) species complex: historical contingency and adaptive radiation |
| 2006 | Kari Mette Murvoll | PhD Biology | Levels and effects of persistent organic pollutans (POPs) in seabirds Retinoids and α-tocopherol –  potential biomakers of POPs in birds? |
| 2006 | Ivar Herfindal | Dr.scient Biology | Life history consequences of environmental variation along ecological gradients in northern ungulates |
| 2006 | Nils Egil Tokle | Phd Biology | Are the ubiquitous marine copepods limited by food or predation? Experimental and field-based studies with main focus on *Calanus finmarchicus* |
| 2006 | Jan Ove Gjershaug | Dr.scient Biology | Taxonomy and conservation status of some booted eagles in south-east Asia |
| 2006 | Jon Kristian Skei | Dr.scient Biology | Conservation biology and acidification problems in the breeding habitat of amphibians in Norway |
| 2006 | Johanna Järnegren | PhD Biology | ACESTA OOPHAGA AND ACESTA EXCAVATA – A STUDY OF HIDDEN BIODIVERSITY |
| 2006 | Bjørn Henrik Hansen | PhD Biology | Metal-mediated oxidative stress responses in brown trout (*Salmo trutta*) from mining contaminated rivers in Central Norway |
| 2006 | Vidar Grøtan | phD Biology | Temporal and spatial effects of climate fluctuations on population dynamics of vertebrates |
| 2006 | Jafari R Kideghesho | phD Biology | Wildlife conservation and local land use conflicts in western Serengeti, Tanzania |
| 2006 | Anna Maria Billing | phD Biology | Reproductive decisions in the sex role reversed pipefish *Syngnathus typhle*: when and how to invest in reproduction |
| 2006 | Henrik Pärn | phD Biology | Female ornaments and reproductive biology of the bluethroat |