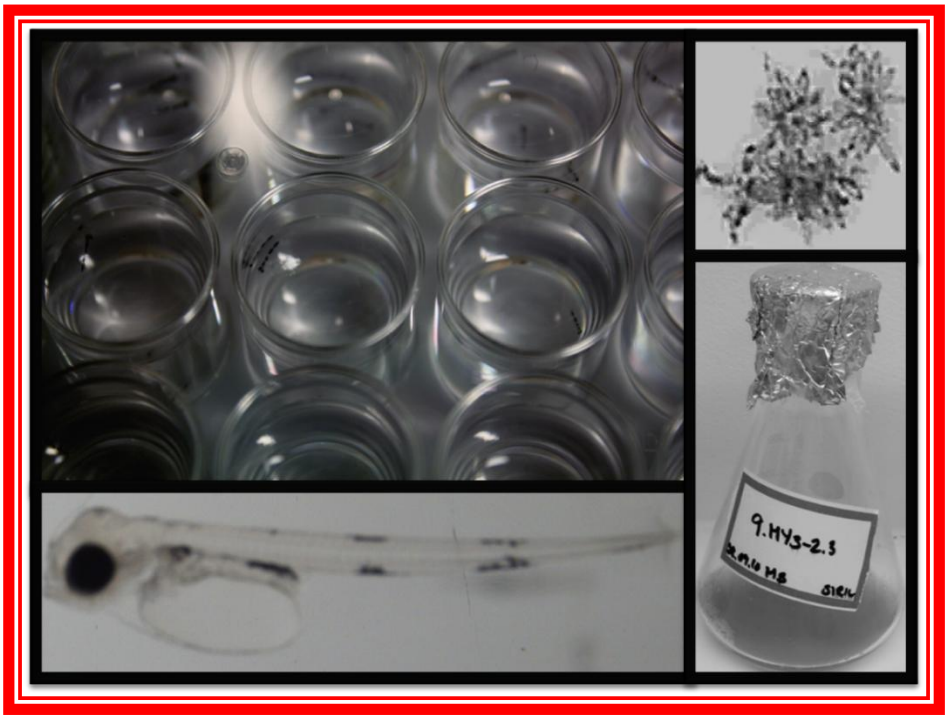
Competition of pathogens and probionts in cod yolk sac larvae measured *in vivo*, and the impact of increased temperature



Thesis for the degree of
Master of Science in Aquamedicine

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Abstract

Aquaculture are today one of the biggest food-producing sectors in the world. Over the recent decades there has been a great increase in intensity and commercialization of aquaculture production, which has led to an unavoidable growth in disease problems. This has again led to a global over consumption of antibiotics and other pharmaceuticals which have caused problems as pollution, resistance and enormous losses for the industry.
Farming of Atlantic cod, *Gadus morhua*, was anticipated to be the new success in Norwegian aquaculture after salmon, but partly due to the global financial crisis, and partly to high mortality – including the early life stages – the success have not become as large as expected. The high density of marine larvae and biological waste during rearing might contribute to high growth of opportunistic pathogenic bacteria, which could result in high larval mortality. Due to the fact that treatments with antibacterial agents are not favourable, and since vaccination is not possible due to the immature immune system of larvae, there has been carried out various studies to find new alternative treatments for the early life stages of cod and other marine species.

The aim of this thesis is to enhance the knowledge of probiotics and test the possibility to use probiotics as an alternative for antibiotics in cod larval rearing facilities.

In the present work a multi-dish system was used as a model for bath challenge experiment, and the species challenged with *Vibrio anguillarum* HI610 and different types of probiotics were cod egg/larvae.
Cod eggs delivered from a commercial hatchery were randomly selected and placed separately in wells in a multi-dish system. Each well is seen as an independent unit and contained 2 ml of 80% aerated sterile seawater. After the eggs were placed in the wells, the wells were challenged with high dose (approximately 10^6 CFU ml^{-1}) of different probiotic strains alone, and together with high dose (approximately 10^6 CFU ml^{-1}) *Vibrio anguillarum* HI610. There were done experiments at 7°C and 13°C (15°C). The experiments did also include a negative control group consisting of unchallenged larvae and a positive control with only high dose *Vibrio anguillarum* HI610. The day hatching reached 50% was defined as day 0, and every day from day 0 and for as long as the experiment carried on, the mortality was registered.

In the present work there were used one pathogen *Vibrio anguillarum* HI610 and there were tested eight probiotics: *Phaeobacter* 27-4, the mutant JBB1001, *Phaeobacter* M23-3.1, *Ruegeria* F1926, *Ruegeria* M43-2.1, *Phaeobacter gallaeciensis* BS107-wt, the mutant *Phaeobacter gallaeciensis* BS107-Pda8 and AQ10 a *Pseudoalteromonas citrea*.

The results are introduced in graphs made in Microsoft Office Excel 2007 showing cumulative mortality in percent (%) per days post hatch (dph) for every challenge group.

The mortality data showed that the pathogenic bacteria *Vibrio anguillarum* HI610 gives a high and rapidly mortality soon post hatch. The probiotics alone did not harm the larvae and could show a slightly positive effect on the normal mortality. The use of the probiotics together with *V.anguillarum* HI610 enhanced an inhibitory effect against the pathogenic bacteria *Vibrio anguillarum* HI610 and/or almost eradication of the effect of the pathogenic bacteria when added at the same time or when the probiotics were added 48hours prior to the addition of the pathogen. Some of the probiotics showed a better probiotic effect than others, and the mutants showed little or no probiotic effect.

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1 Introduction

1.1 The Atlantic cod

There are several stocks of Atlantic cod, distributed in different areas. Depending on the stock and the distribution, Atlantic cod can become up to 1,3meter and 40kilos (Holm, 1999). The Atlantic cod is widely distributed in the North Atlantic Ocean. In the western Atlantic it is found at the east coast of America to Cape Hatteras, at both sides of south Greenland and all around the coast of Iceland. In the east, it is found from the Bay of Biscay in the south to the northern part of the Barents Sea (Svåsand et al., 2004). Over the years there has been a decline in the wild stocks comparing with historical levels, and in the mid 1980s there was a collapse of cod population in the Barents Sea due to a lack of juvenile herrings and reduced capelin stock (Hamre, 1994). In 1992 the fisheries for Atlantic cod in some areas of Canada were stopped (Svåsand et al., 2004). Because of the decline in the wild stocks, the North Sea Cod and the Costal Cod south of 62°N have been included to the list of endangered populations in Norway and to the list of species needing special precaution by the Norwegian environmental authorities, Directorate of Fisheries (Anonymous, 2009). Cod as a species is not listed, but many stocks are endangered with declined stocks, little recruiting and the absence of proper regulation and precautions (Storeng, 2010). Despite that the natural cod stocks are small and endangered compared to the historical levels, the total stock and spawning stock of the Northeast Arctic Cod is increasing and the spawning stock were estimated to be around 1 350 000tonnes in 2010, which is over the limit set by the Directorate of Fisheries (Sunnana et al., (red.) 2010).

Atlantic Cod is one of the most important fish stocks in Norway for commercial fisheries and export. In 2010, the Directorate of Fisheries reported that 283 312tonns were caught, about 40tonns more than in 2009 (Anonymous, 2011a). The Norwegian Seafood Export Council could report an increase of exported value of cod from May 2010 to May 2011 by 768 million NOK or 12% due to the quota increase (Anonymous, 2011b).

1.2 Aquaculture of cod

As early as in the 1880s it was showed that artificially hatched cod larvae were viable. These trials were conducted by Captain G. M. Dannevig in Flødevigen, Arendal, where he developed and used extensive (poll-) methods. This program was financed by public funds to strengthen the natural population of cod. This method was used for a long time despite there were no proofs of any benefits (Svåsand et al., 2004, van der Meeren and Naas, 1997). In the 1970s, when the Flødevigen project was terminated, new efforts were put in to develop the extensive and semi-intensive systems, but the production results were to small and unpredictable. A hundred years after the first attempt with artificially hatched cod larvae, the development and use of intensive production took shape in the 1980`s. The live-food-culture based production on cod started. However, unpredictable production, low prices and year depending production resulted in poor outcomes and there were low interest. Around the late 1990s and early 2000s there was an increase in the interest for cod larvae production due to reduced stocks and poor catches of wild cod, and also good development for the salmon farmers and few salmon cultivation permits available (Svåsand et al., 2004). Every year from 1999 the production and sale of Atlantic cod has increased, until it reached a top in 2010. This has been achieved in spite of all the challenges this small part of the aquaculture industry has had to conquer. Some of them are:

- low prices for finished product,
- high production cost,
- financial crises,
- investors giving up,
- few juvenile producers left,
- challenging with diseases and
- too early maturation.

However, still the commercial cod aquaculture is not profitable, some due to the high production cost and the low prices per kilo sold cod. The average price per kilo cod sold in 2010 was 16,36NOK (Anonymous, 2011c) and the price to produce 1kilo cod filet, costs in average 30NOK (in 2009) (Eliassen, 2009). This comparison shows that there are still major obstacles for this industry to overcome.

1.3 Immunology of cod larvae

Fish eggs hatches on different ontogenetic stages, some more primitive and less developed than others. Cod can be viewed as an intermediate case with respect to developmental stage at hatching, and the immune system of cod is not fully competent until 2–3 months after hatching (Magnadóttir et al., 2004, Schrøder et al., 1998, Vadstein et al., 2004). This means that before this stage, the specific immunity is not fully advanced and the larvae are fully reliant on their non-specific defence against infections. The non-specific defence or innate immune system is regarded as the first line of defence, and also probably the major defence against micro-organisms in larvae. The parameters of the non-specific defence do not require prior contact with a pathogen to elucidate a response (Vadstein et al., 2004). These parameters are the complement system, various lectins, lytic enzymes, antibacterial peptides, proteinase inhibitors and phagocytes (Magnadottir et al., 2005). The phagocytic activity is mainly associated with the gills, skin and gut. This is also the areas where the larva first comes in contact with pathogens. The gut is closed the first days post hatch, but the larvae are able to drink water via the intestine, as the pseudobranch is open. The specific immune system does require activation and a time delay before the protection is achieved. It is important that the immune system, especially the specific, is fully developed before vaccination, because too early vaccination may lead to reduced survival due to immunosuppression rather than immunoprotection (Vadstein et al., 2004).

New important studies performed by the Centre for Ecological and Evolutionary Synthesis (CEES), Department of Biology, University of Oslo in cooperation with other Norwegian university and Norwegian research institutions have sequenced the genome for the Atlantic cod (*Gadus morhua*). The presentation of the genome sequence reveals that the cod genome is completely without the MHC II- genes, that notifies the immune system about infections by bacteria, parasites and fungi in all known vertebrates. The genome also lacks other immunresponse genes, making the cod genome characteristic and questioning the fundamental assumptions about the evolution of the adaptive immune system and its components in vertebrates (Star et al., 2011). These new revelations will probably give the immunology of cod larvae a new “structure”.

1.4 Bacterial diseases in cod

Compared to a life in air, a life in water especially seawater is more hostile when it comes to microbial interactions. A fish has to handle bacterial concentrations of approximately 10^6 bacteria per ml^{-1} seawater, and the concentration is considerably higher in environments with higher input of organic matter, like in aquaculture systems (Vadstein et al., in press 2011 , Vadstein et al., 2004). The high bacterial concentration is also the case in cod aquaculture and the bottleneck for this industry is especially the intensive production of egg and larvae (Svåsand et al., 2004). The intensive production of egg and larvae gives good growth conditions for bacteria some of it is because of the mucosal surface of eggs and larvae that have good adhesion for bacteria (Hansen and Olafsen, 1989). Bacteria that are dominant in the epiflora on eggs and early yolk sac period are mainly from the groups *Flexibacter*, *Flavobacterium* and *Cytophaga*, while *Vibrio* spp. are not that frequent (Hansen and Olafsen, 1989) Disinfection may resolve many problems the bacteria on the egg surfaces might cause. At the time larvae starts feeding the microbial flora may have changed a lot, new challenges and new massive inflow of bacteria follows the alive feed and the organic waste from feeding, like *Vibrios* and *Areomonas* (Brunvold et al., 2007, Korsnes et al., 2006, Vadstein et al., 2004).

The biggest challenges of bacterial infections for the adult cod in the farming industry in general are classical vibriosis, *Vibrio anguillarum,* a-typical furunculosis, *Aeromonas salmonicida* and francisellosis, *Francisella noatunensis*. In 2010 there were proofs of vibriosis from *Vibrio anguillarum* in 6 locations, 5 locations showed proofs of a-typical furunculosis and 3 locations showed proofs of francisellosis, this is a decrease from earlier year's registrations. The decrease is probably because of the decrease in numbers of active cod facilities, the decrease in samples sent to the Norwegian Veterinary Institute for examination, and that the active rearing sites are sited further north were the temperature is lover and less favourable for these pathogens (Hellberg, 2010).

1.5 Vibrio anguillarum

Vibrio anguillarum is probably the one bacterial fish disease that has been known the longest, as early as in 1718 there was a description of a disease called "red-pest" in eels. Later on in 1893 Canestrinin gave the bacteria causing the "red-pest" disease the name *Bacterium anguillarum*, before Bergman in 1909 re-named it *Vibrio anguillarum* (Austin and Austin,

2007, Larsen and Pedersen, 1999). *Vibrio anguillarum* are Gram negative bacteria in the family Vibrionaceae and in the genus *Vibrio*. The genus *Vibrio* is mainly marine bacteria except from a few human and other vertebrates and invertebrates pathogenic bacteria. In the mid-80s due to 5s rRNA sequence analysis *Vibrio anguillarum* were reclassified as a member of the family *Listonella* by (MacDonell and Colwell, 1985), cited by (Holt and Bergey, 1994, Isachsen, 2009, Smith et al., 1991) but there is still some debate regarding this change in nomenclature so both names *Vibrio anguillarum* and *Listonella anguillarum* are valid today. I will use the name *Vibrio anguillarum* in this thesis.

Vibrio anguillarum is a gram negative, straight or curved shaped rod with polar flagella, it is oxidize-positive, has optimum temperature for growth between 15-37°C and it requires addition of salt in the growth medium (Austin and Austin, 2007, Larsen and Pedersen, 1999). Iron accessibility plays an important role for the virulence of *Vibrio anguillarum* (Larsen and Pedersen, 1999). *V. anguillarum* is a heterogeneous species divided into serotypes, and at least 23 O serotypes are described today (Pedersen et al., 1999). Serotype O2a and O2b are the ones associated and found in cod aquaculture (Hellberg, 2010).

1.6 Vibriosis

Vibriosis is a generic term for infections caused from different *Vibrio* species. In the marine fish, cod (*Gadus morhua*), it is the species *Vibrio anguillarum* that causes the specific infection, classical vibriosis, a classical gram negative hemorrhagic septicaemia that often occurs when water temperature rises quickly. When the disease, classical vibriosis is acute it gives acute hemorrhagic septicaemia, and the fish dies without any externally visible clinical signs. When it is sub acute/chronic it gives ulcerous hemorrhagic septicaemia with ulcer and fin rot. Typically outer clinical signs with a vibriosis infection are bleedings in the skin, around mouth, and on fins, ulcer, fin rot, exophtalmus and formation of abscesses. Typically inner clinical signs are bleedings in the peritoneum, bleedings and necrosis in the liver, swelled spleen and kidney, and ascites (Austin and Austin, 2007, Larsen and Pedersen, 1999).

Many serotypes of the different *Vibrio* species exist. In the case of *Vibrio anguillarum*, serotypes O2a and O2b are known to cause outbreak of vibriosis in cod aquaculture (Hellberg, 2010). When there are bacterial infections the treatment used is antibiotics, before feeding it is usually bath-treatment. When the larvae feeds formulated feed the treatment can be administered orally by medicine pellets, if the appetite is any good, if not bath-treatment is

used (Samuelsen et al., 2006). Still there is yet not been registered any resistance against the used antibiotics that are used (Hellberg, 2010). However, water quality and good farming and not to high biomass could probably to a certain extent prevent stress and possible disease outbreaks. Vaccines are important tools to prevent infections, however, they cannot be used until the larvae are big enough and the immune system is fully developed. Vaccines against *Vibrio anguillarum,* vibriosis in salmonid fish have existed for almost 20 years with success, but the vaccine against vibriosis in cod that have existed for 10 years do not provide the same sufficient protection, so vibriosis is still a problem for the cod farmer (Samuelsen et al., 2006, Sommerset et al., 2005). In 2010 there were proofs of vibrio in 6 locations with cod farming. This is a major decrease from 2009 where there were proof of vibrio in 16 locations and in 2008 when there were proofs of vibrio in 20 locations. This major decrease in outbreaks could have been positive, however, it must be seen in connection with the high reduction in active cod farms the recent years, and that the remaining locations are sited further north than earlier, with cod at lower temperatures possibly being less susceptible to *Vibrio* (Hellberg, 2010).

1.7 Probiotic

Probiotic means "for life" and originates from combining the Latin word pro (for) and the Greek word bios (life) (Zivkovic, 1999). Probiotics were first reported in 1908 by Elie Metchnikoff. Elie Metchnikoff observed that a large number of people in Bulgaria became more than 100-years old and linked this observation to large consumptions of yoghurt. He isolated bacteria from the yoghurt and concluded that the bacteria were the cause of a health promoting effect (referred in (Gillor et al., 2008)). Throughout time probiotics have had many definitions. The first widely accepted definition of probiotics for warm-blooded animals and the one definition most quoted was *"a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance"* (Fuller, 1989). This definition associates the probiont with feed, but in aquaculture the culture water is also an important medium for exposing the fish to the probiont (Gomez-Gil et al., 2000). This resulted in several definitions trying to fit the aquatic environment and organisms (Gatesoupe, 1999, Gram et al., 1999). In the most recently accepted definition, probiotics are defined as: *"live microorganisms which when administered in adequate amounts, confer a health benefit on the host"* (FAO and WHO, 2001). This definition is probably the most commonly quoted,

probably the most suitable for all environment including the aquatic and the one used in this thesis.

The probiotics modes of action are: I) competition for attachment sites, II) competition for nutrients or chemicals and III) production of antagonistic compounds. However, it is also very likely that the mode of action is a combination of several mechanisms (Fuller, 1989, Porsby, 2010, Verschuere et al., 2000b).

I) Competition for attachment sites are a competition between a probiotic and a possible pathogen for an adhesion site on gut or other tissue sites. Such competition could prevent colonization and infection of pathogens (Porsby, 2010, Verschuere et al., 2000b). One example is given by (Vine et al., 2004) who found that a possible probiotic bacteria isolated from clownfish (*Amphiprion percula*) was able to prevent attachment of *Vibrio alginolyticus* and remove the pathogen from the surface. Also some studies have been done on biofilm making probiotics, probiotics that need to form biofilm to be effective probiotics (Bruhn et al., 2006, Brunvold, 2010, Hjelm et al., 2004, Porsby et al., 2008). Thus this is not directly attachment competition, it is rather about surface attachment and "getting in there first" (Irianto and Austin, 2002).

II) Competition for nutrients or chemicals is probiotics that can interfere with the composition of the microbiota in culture water or on surfaces on the host. The microbiota is generally dominated by heterotrophs, which compete for organic substrates as carbon and energy sources (Verschuere et al., 2000b). There is no research that can document the competition for nutrients for a fact, but there are researches where they use empirical approaches and conclude with a competition theory (Rico-Mora et al., 1998, Verschuere et al., 2000a). Competition for iron has showed to be a possible inhibitor for pathogens. Iron is largely unavailable since it is limited in the tissues and body fluids of animals and in the insoluble ferric Fe^{3+} form (Verschuere et al., 2000b). Most bacteria need iron for growth and iron-binding agents called siderophores allow acquisition of iron suitable for microbial growth. Production of siderophores and uptake of iron are mechanisms of virulence in some pathogens (e.g. *Vibrio anguillarum* (Tolmasky and Crosa, 1991)) and if a probiotic uses siderophores as a mode of action it will clear the environment for iron and thereby limit growth of pathogenic bacteria (Gram et al., 1999).

III) Production of antagonistic compounds, also called inhibitory compounds is production of chemical compounds by microbial organisms that have a bactericidal or bacteriostatic effect on other microbial organisms. If such antagonistic producing organisms are present in rearing waters, in intestine or surfaces of the host it is believed that they could either prevent

proliferation of pathogenic bacteria, or kill them (Porsby, 2010, Verschuere et al., 2000b). Examples of factors that in general give antibacterial effect are: production of antibiotics (Williams and Vickers, 1986), bacteriocins (Bruno and Montville, 1993, Pybus et al., 1994, Vandenbergh, 1993), siderophores, lysozymes, proteases, and/or hydrogen peroxide, ammonia and diacetyl (Vandenbergh, 1993) and the alteration of pH values by the production of organic acids (Sugita et al., 1997). If antagonistic compounds are the mode of action of probiotics that outcompete different pathogens, the possibility of developing resistance against these compounds has to be taken into account just like resistance for antibiotics, and experiments needs to be done.

There have been many *in vitro* experiments to test different bacteria antagonistic effects against fish pathogens (Gatesoupe, 1999, Gram et al., 1999, Hjelm et al., 2004, Robertson et al., 2000), but only a few possible probionts have been tested in *in vivo* challenge trails (Gram et al., 1999, Planas et al., 2006, Porsby et al., 2008), therefore more tests have to be carried out for the preference bacteria/probiont.

One group with potential probiotics that lately have received increasing attention in marine aquaculture, are the so-called *Roseobacter* clade and include 38 different genera (Brinkhoff et al., 2008). Bacteria in the *Roseobacter* clade are commonly found in marine environment (Moran et al., 2003, Wagner-Döbler and Biebl, 2006) and *Phaeobacter* spp. and *Ruegeria mobilis*, which belong to the *Roseobacter* clade, are to be found in marine larval rearing systems. These bacteria are known to inhibit growth of or kill different fish pathogenic bacteria *in vitro* (e.g. *Vibrio anguillarum*) and have shown to reduce mortality of fish larvae infected with pathogenic bacteria (Hjelm et al., 2004, Porsby et al., 2008). It has been suggested and tried in challenge trials that it is TDA, a secondary metabolite tropodithietic acid, which work like an antagonistic compound and inhibit the pathogen growth for some *Roseobacters* (Brinkhoff et al., 2004, Bruhn et al., 2005, Porsby et al., 2008). It has been showed that the production of TDA is related with stagnant growth conditions, the ability to grow in rosette-like structures and production of brown pigment. The *Ruegeria* strains were not able to display these phenotypes at shaking conditions as in stagnant condition like the *Phaeobacter* strains could, except *Phaeobacter* strain 27-4 that behaves like a *Ruegeria* (Bruhn et al., 2005, Hjelm et al., 2004, Planas et al., 2006, Porsby et al., 2008). There have been performed challenging trials that showed that it most likely not will occur any resistance among pathogens against TDA and it showed that TDA is bactericidal against both Gram-negative and Gram-positive bacteria (Porsby, 2010). These results make TDA producing bacteria interesting for control of unwanted bacterial growth in aquaculture.

1.8 Probiotics in aquaculture

Vaccination has shown to be a successful protective immunity against fish- pathogen, but for fish larvae, crustaceans and molluscs the use of vaccines against pathogens are not possible. Because of this, and that antibiotics are not favourable because of the risk for resistant bacteria and pollution in the environment, the possible effects of using probiotics has been the focus of several experiments (D'Alvise et al., 2010, Gram et al., 2010, Planas et al., 2006).

For some extent probiotic in formulated feed (Robertson et al., 2000) and in live feed, artemia and rotifers (Harzevili et al., 1998), have already been in use in aquaculture. Also probiotics added directly in the rearing water have been tried (Austin et al., 1995, Ringø and Birkbeck, 1999). Using probiotic bacteria in aquaculture seems like a promising way to control diseases and there have been showed good effects in several experiments, especially in molluscs rearing, but there is still much work and research that needs to be done before probiotic can be used commercially with documented economics effects. In order to aid in correct establishment of new, effective and safe products there has been proposed some (Kesarcodi-Watson et al., 2008, Verschuere et al., 2000b) properties that the probiotic should possess, these are:

- the probiotic should not be harmful to the host it is desired for,
- it should be accepted by the host, for example through ingestion and potential colonization and replication within the host,
- it should reach the location where the effect is required to take place,
- it should actually work *in vivo* as opposed to *in vitro* findings,
- it should preferably not contain virulence resistance genes or antibiotic resistance genes.

These properties could be incorporated into an overall including question: “does the potential probiotic provide an overall health benefit when given to the animal?” This must be proved out by experiments that prove the different probiotics benefits to the host: Experiments *in vivo* as well as *in vitro*, and experiments that proves the ability the probiotic has to colonise, and in a positive way effect the microbiological environment in the gastrointestinal tract (Kesarcodi-Watson et al., 2008).

In addition to the different probiotics actuall effect/benefit, the delivery routes used for adding the probiotic organism to the aquaculture rearing system has to be improved for what the different probiotic requires, and for which host and pathogen the probiotic works for. Today

the delivery routes are : Bath of the host in a suspension of the probiotic organism, addition of the probiotic organism to the culture water in the tanks, administered as addition to the artificial diet and addition via live feed (Verschuere et al., 2000b). The addition direct to the water are only possible for larva stages or other situations with tank rearing or low-flow-through rates (Makridis et al., 2000b, Ringø et al., 1996, Ringø and Vadstein, 1998), and encapsulated in feed is the only possible method of administration in open or high-flow-through systems. The administration of probiotic through encapsulated feed only works for the probiotic where the strains can be kept dry for a while or where the probiotic strains can be added/fed via live feed (Gatesoupe, 1999, Makridis et al., 2000a). Bath/suspension of the host in a suspension of the probiotic organism is rarely used due to the stress on the host, and the amount of bacteria that would have to be cultivated. All these routes require validation because of the differences between various strains of bacteria and because of the variation among the different cultured species.

1.9 Global warming/climate change

The terms global warming and climate change are often used synonymously, but have different meanings. While “global warming” are commonly used on humanly caused alterations by discharge of greenhouse gases, that among others leads to acidification of the environment and changes in temperature, climate change is statistical variation in the global or regional weather, as temperature or precipitation, lasting over periods for months, years, decades or more (Nodvin, 2010, Anonymous, 2011e).

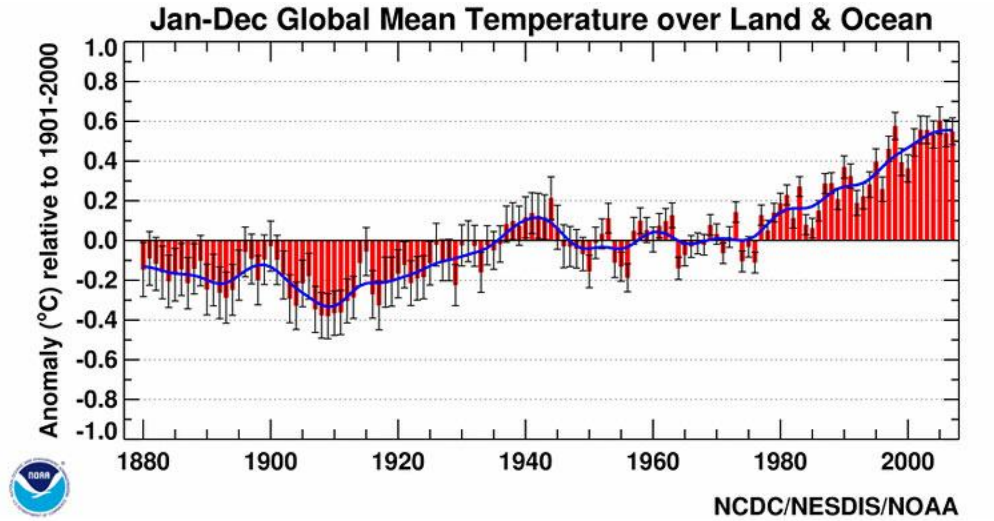

Figure 1.9.1: Shows the Global Mean Temperature over Land and Ocean (Jan-Dec) from the 1880's until 2009, presented of NCDC/NESDIS/NOAA at the internet page The Encyclopedia of Earth (Nodvin, 2010). http://www.eoearth.org/article/Global_warming?topic=49491

Measured data illustrated in figure 1.9.1 made by the NCDC (National Climatic Data Center, U.S Department of Commerce), NOAA (National Oceanic and Atmospheric Administration) and NESDIS (The National Environmental Satellite, Data, and Information Service) presented at the internet page to The Encyclopedia of Earth (Nodvin, 2010) shows that from the late 1800's until 2009 there have been an increase in the global mean temperature, and the decade 2000 (2000-2009) shows the warmest temperatures on record. These temperature data is presentable with figure 1.9.2 that the Norwegian Meteorological Institute present on their internet page (Anonymous, 2011d) that shows an increase of the Norwegian temperature over the last 100 years.

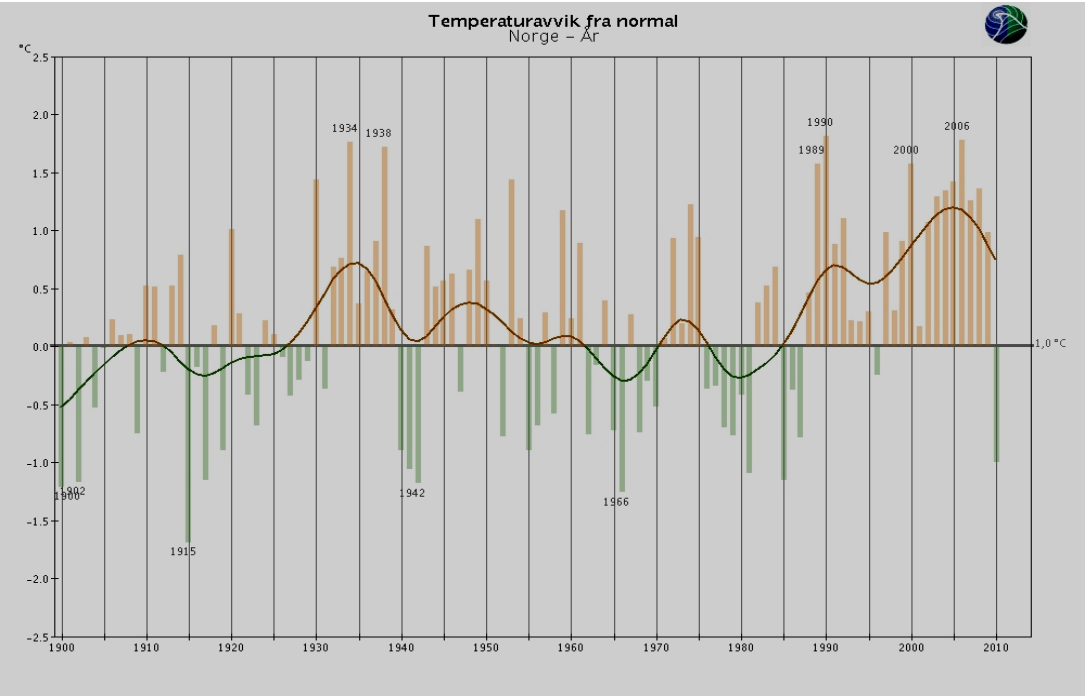

Figure 1.9.2: Shows the smooth temperature increase in Norway from the beginning of the 1900's and until 2010. Years that deviate from the normal, are marked. (Anonymous, 2011d). http://met.no/Klima/Klimautvikling/Klima_siste_150_ar/Hele_landet/

These climate changes and the global warming will have an impact on the global ocean temperature and environment. During the 21st century the temperature at the sea of the coast of Norway is expected to rise with 1,5-2,0°C and the water environment will, because of the high CO_2 and other greenhouse gas emissions, become more acidified (Bergh et al., 2007). These changes will again have significant effect on the aquaculture industry and aquaculture locations might have to relocate or modify the farming technology to fit the changing environments. The higher temperatures will force the fish farmer (especially in the south) to move the locations further north to get lower temperatures (particular in the summer months). In addition the salinity in the fjords might decrease and there will become a clearer stratification between freshwater and saltwater due to increased rainfall and higher levels of runoff from land, forcing the fish farmer to move the locations further out from the fjords and into open sea. Further on, the climate change might lead to extreme weather and storm activity that could damage the fish farms and more fish could escape (Bergh et al., 2007).
A temperature increase would also cause a biological impact on the different fish species in aquaculture, some as benefits and others as disadvantages.

As higher temperature have shown reduced disease resistance and many fish pathogens have a higher optimum temperature than the fish in the Norwegian aquaculture, it is natural to think that the climate change with a temperature increase will create more outbreaks of diseases. Good examples are the fish diseases furunculosis and vibriosis (vibriosis chemotactic and optimums temperatures (Larsen et al., 2004)) that often occurs in the summer months and early autumn. Higher temperature reduces the amount of oxygen in the water, and this combined with high biomass increases the stress level, which again reduces disease resistance (Bergh et al., 2007).

The temperature around hatching is important for the embryo development and variation over or under optimum could give high frequency of deformed larvae. The tolerance for higher temperature is dependent on natural habitat and species.

Some temperatures experiments have been performed to find out the temperature column for optimum growth of cod (Moksness et al., 2004, Otterlei et al., 1999), and these have showed that growth is dependent on temperature, but also on feed availability and size. Similar results apply to the immune system, which becomes less functional outside certain species-dependent temperature limits (Bowden, 2008, Bowden et al., 2007).

1.10 Background and aim

Atlantic cod, *Gadus morhua*, has become an important species in Norwegian and European aquaculture. But the production has been limited by unstable production of juveniles due to high mortality in the early life stages (Bricknell and Dalmo, 2005, Samuelsen et al., 2006). The high mortality is partly caused by opportunistic pathogenic bacteria and since the immune system of larvae is so immature, prophylactic and/or therapeutic use of antibiotics is the main option for protection against bacterial infection. Due to the increase of antibiotic resistance in bacteria the use of probiotics has been proposed as a possible substitute for preventing or reducing bacterial diseases (Gatesoupe, 1999, Hjelm et al., 2004, Holzapfel and Schillinger, 2002, Irianto and Austin, 2002, Verschuere et al., 2000b).

The general aim of this study was to enhance our knowledge of a probiotic used as a health benefit for the cod larvae and as an antagonist against *Vibrio anguillarum*.

The ultimate test and further research, when one know for sure that the probiotic is not harmful, would be big field trials or trials at a rearing facility for cod or halibut. One will also

need to find a reasonable way of cultivating the probiotic in large scale and a method or pump system to add the probiotic to the rearing water.

Specific aims for this study were:

- To perform *in vivo* competition experiment with cod yolk larvae between a range of tentative probiotic bacteria and one pathogen, *Vibrio anguillarum* strain HI610, modelled from Hjelm et al. (2004) but with cod larvae instead of turbot larvae.

- To add a temperature gradient (13°C and 15°C) to the *in vivo* competition experiment in order to describe the impact of temperature increase on the pathogen-probiont competition. This will be done with *Vibrio anguillarum* based on the assumption that this pathogen is associated with high temperatures (Bergh, 2007, Samuelsen et al., 2006), and that the immune defence is affected by temperature (Bowden, 2008, Bowden et al., 2007).

- To register mortality against days post hatching in all groups: pathogens alone, probiotic alone, pathogen and probiotic together, probiotic first and pathogen later, pathogen first and probiotic later, and the same groups but at different temperatures. To perform statistical calculations to emphasize or eliminate significantly differences between groups.

2 Materials and methods

2.1 Eggs

Eggs were collected from the breeding programmes of two commercial cod hatcheries, named hatchery A and B in this thesis. The origin of the broodstocks was coastal cod and "skrei", (Northeast Arctic Cod). Before transferred to our lab at Høyteknologisenteret in Bergen, the eggs from hatchery A delivered in time for experiment 4 and 5 were treated with Buffodine (Evans Vanodine International Plc), disinfection used for fish eggs, while there was no treatment with disinfection agents at the eggs for experiment 3, delivered from hatchery A, and at the eggs from hatchery B, delivered in time for experiment 2 and 5.

Transportation of the eggs from hatchery A to Høyteknologisenteret in Bergen took around 4-5 hours in total, by boat and by car. The transport of eggs from hatchery B took in total 3-4 hours by plane and by car. The eggs were transported in polystyrene containers with cooling elements and the average temperature under transport was around 8°C.

2.2 Bacteria

The bacteria used in these challenge experiments were: The *Vibrio anguillarum* strain, HI610 a serotype O2α, which was used as a positive control in these experiments. The strain was originally isolated from vibriose-infected cod juveniles from the closed seawater basin at The Institute of Marine Research in Parisvatnet, Øygarden, Norway. The strain has been routinely stored in the culture collection at -80 °C at The Institute of Marine Research until the experiment started, and has previously been used in a range of challenge experiments (Samuelsen and Bergh, 2004, Seljestokken et al., 2006, Vik-Mo et al., 2005).

The probiotics that were tested in these challenge experiments were mainly from the genera *Phaeobacter* and *Ruegeria* (clade *Roseobacter*). These probiotics have been used and tested in earlier experiments (Hjelm et al., 2004, Porsby et al., 2008)

One of the probiotics used in all the challenge experiments, *Phaeobacter* 27-4 is known to enhance the survival of turbot egg yolk sac larvae and to be highly active against several *Vibrio* spp. (Hjelm et al., 2004, Planas et al., 2006). It has been showed that *Phaeobacter* 27-4 behaves more like a *Ruegeria* because it needs static condition to grow in rosette shape and

make brown pigment, unlike the other *Phaeobacter* strains tested in these challenge experiments (Bruhn et al., 2006, Bruhn et al., 2005, Hjelm et al., 2004, Porsby et al., 2008). *Phaeobacter* 27-4 was isolated from a turbot larvae (*Scophthalmus maximus*) rearing unit in North Western Spain during a study of selection and identification of autochthonous potential probiotic bacteria (Hjelm et al., 2004). It has like the *Vibrio* strain been kept in the culture collection at -80 °C at The Institute of Marine Research until experiment started.

Phaeobacter strain JBB1001 is a *tdaB* mutant of *Phaeobacter* 27-4 and do not produce hampering substance TDA, tropodithietic acid that is a secondary metabolite that is likely to cause the antagonistic effect against other pathogenic bacteria. TDA is known as an antimicrobial in aquaculture (Geng et al., 2008).

The probiotic *Phaeobacter* M23-3.1 was also used. This strain was isolated from a Danish turbot larvae rearing unit, and it has been shown to be highly active against *Vibrio* (Hjelm et al., 2004, Porsby et al., 2008).

Ruegeria M43-2.3 was another strain that likewise with *Phaeobacter* M23-3.1, was isolated from a Danish turbot larvae rearing unit, and has also shown to be highly active against *Vibrio* (Hjelm et al., 2004, Porsby et al., 2008).

Ruegeria F1926 was isolated in the Indian Ocean at the Galathea-expedition (Gram et al., 2010, Porsby, 2010)

Later in the experiment I received two *Phaeobacter gallaeciensis* strains that we used in the third, fourth and fifth challenge experiment. One was the *Phaeobacter gallaeciensis* BS107 wt, the wild strain. The other was a TDA deficient mutant of the strain, *Phaeobacter gallaeciensis* BS107-Pda8. (Transposon-insertion mutant) The wild type has been demonstrated to be able to inhibit growth of, or kill a variety fish pathogenic bacteria (Ruiz-Ponte et al., 1998). While the mutant that lack TDA was used like a positive control, to show that it is TDA that works like a pathogen inhibitor. All these probiotic strains were received from The Technical University of Denmark, the department DTU Food, by Professor Lone Gram.

In addition to the seven probiotics there were also challenged and made growth curve with a bacteria called AQ10, a *Pseudoalteromonas citrea*. This bacterial strain was isolated from a wall surface of a sea-water pool in the public Bergen Aquarium (Norway) and have since then been kept at 20% glycerol and at -80°C at The Institute of Marine Research until the experiment started. AQ10 has showed a strong antagonistic activity against some marine pathogens, and one of them is *Vibrio anguillarum* (Brunvold, 2010, C. Lambert, IFREMER, Brest; France, unpublished data)

Table 2.2: Overview of the different bacterial strains used in these experiments, the species or place from which they were isolated and references.

Bacteria	Origin	References
Vibrio anguillarum strain HI610	Cod larvae	(Samuelsen and Bergh, 2004, Seljestokken et al., 2006, Vik-Mo et al., 2005)
Phaeobacter strain 27-4	Turbot larvae	(Hjelm et al., 2004)
Phaeobacter strain M23-3.1	Turbot larvae	(Hjelm et al., 2004, Porsby et al., 2008)
Phaeobacter gallaeciensis BS107-wt	Scallop larvae	(Ruiz-Ponte et al., 1998)
Phaeobacter gallaeciensis BS107-Pda8	Scallop larvae	The Technical University of Denmark, the department DTU Food, and Professor Lone Gram's program
Ruegeria strain M43-2.3	Turbot larvae	(Hjelm et al., 2004, Porsby et al., 2008)
Ruegeria strain F1926	in the Indian Ocean	(Gram et al., 2010)
Phaeobacter mutant JBB1001	Turbot larvae	(Geng et al., 2008)
Pseudoalteromonas citrea AQ10	Wall surface Bergen Aquarium	(Brunvold, 2010)

2.3 Cultivation and growth curve

Cultivation and measurement of the growth curve of the *Vibrio anguillarum* HI610 were carried out by Kristian Dam, Department of Biology, University of Bergen, Norway. (More information about the procedure in Appendix 7.3)

Cultivation of the probiotics was based on methods used in the PhD Thesis by Porsby (2010). The probiotics were cultivated in 20ml Marine Broth in 250ml Erlenmeyer bulbs, in an incubator (Memmert GTR0214) at 20°C and in the dark without shaking.

The growth curves for all the probiotics were set up based on the master thesis by Kolstø (2008), who described a method for growth curves for *F. philomiragia* subsp. *noatunensis,* and further development and adjustment were made by me and my supervisor to fit the growth of the probiotics.

A preculture was made by inoculating bacteria, that were grown on agar plates, to a 250ml Erlenmeyer bulb with 20ml Marine Broth. The bulb was covered with tinfoil and stored for 5 days without shaking at 20°C. After 5 days, 2% volume/-volume from the preculture were transferred to 10 (17 for P.g wt and P.g Pda8) 250ml Erlenmeyer bulbs with 20ml Marin Broth. These bulbs were also covered in tinfoil before they were stored at 20°C in an incubator without shaking, until it was time to measure. Before measuring, one bulb were taken out of the incubator and well shaken before 1000µl of the culture were putted over in a cuvette (Semi-micro, PS, Styrofeam raek with lid of 100, Germany) and the optical density, OD, of the bacterial cultures was measured at 600 nm in a Hitatchi U-1100 Spectrophotometer. The OD was measured each 12 hour for 4 days, using a new bulb for each measuring, and the last bulb with bacteria suspension was measured at day 7.
At three points in the growth curve, a CFU, Colony Forming Units or plate-count was made. This was done in order to have a growth curve based on two different varieties. However, plate-count turned out to be problematic due to the probiotics stickiness and rosette formations, causing underestimation of cell numbers, discussed under Discussion. The results of the growth curve are shown in growth curves figures 3.1 a) to 3.1 i) under Results.

2.4 Plate-count, CFU or Colony Forming Units

Plate-counts of CFU or Colony Forming Units are a way to estimate a concentration of viable bacteria in a suspension. CFU estimate were made by a series of dilutions were autoclaved distilled water with 2%NaCl was used as dilution medium. Seven or eight ten-fold dilutions were made. From the three rearmost dilutions tubes three times 100µl on (in total) 9 Marine agar -plates was plated out. The Marine agar -plates with diluted suspension were then placed in the incubator (Memmert GTR0214) at 20°C for 3 days. Plates with 50 to 150 colonies were counted and the CFU per ml of culture were calculated by using the following formula:

$$Number\ of\ cells(CFU)per\ ml = \frac{Numbers\ of\ colonies(CFU)pr\ plate}{Dilution\ Factor}$$

The results for the growth curves and the challenging dose in the challenging experiment showing OD and CFU are showed in figures 3.1 a) – 3.1 i) and tables 3.2 a) – 3.2 c) under Results, and in table 7.8 a) and b) in Appendix 7.8.

2.5 Counting bacteria

The first time the challenge doses were defined, and the first time the growth curves were implemented, a Tiefer counting chamber (depth 0,02mm, square-net) was used to decide the concentration of bacteria in the cultures.

The bacterial suspension until one had 10 cells in each "small square" or 60-70 cells in each "big square", often a 1:10 dilution. To make sure that there were no other objects that could derange the counting, the counting chamber and cover-slip was cleaned with 70% lab alcohol. The cover-slip was putted over the square-net and one drop of diluted bacterial suspension was putted at the side of the cover-slip so the capillary-force dragged the suspension under the cover slip. A Nikon Alphaphot-2 YS2 microscope was used to find the square-net and to count the bacteria. The number of squares and the numbers of bacteria were counted until 200-300 bacteria had been counted.

The number of counted bacteria was divided on the number of counted squares. This gave the number of bacteria in each square and this number was then multiplied with the volume for one cell in each square, and one got the total number of bacteria in the diluted suspension. This answer multiplied with the times the suspension was diluted, gave the number of bacteria for each millilitre in the original bacterial suspension, the concentration of bacteria in the suspension. The counting chamber method had some uncertainties because dead and alive cells are both counted.

This method cannot be used to count the probiotics like *Phaeobacters* or *Ruegeria* because they cluster together in rosettes with individual cells that can be hard to distinguish. It was not judged a good method for counting the AQ10 either, even though they were not forming rosettes, they were too small to distinguish properly with the equipment that we used.

2.6 Optical density in NaOH, lye

Since the probiotics that were used were making rosettes and were difficult to separate, the counting chamber technique was inappropriate. Thus, another method to decide the concentration of the challenge doses was needed.

The bacteria were washed and diluted as when the challenge doses were made. Then 1,5ml of the diluted bacterial suspension was added to a 1,5ml Microtube (AXYGEN) and centrifuged in an Micro centrifuge (Beckman Microfuge, Lite Centrifuge) at $12min^{-1}$ (x1000) for 10 minutes. After centrifugation the suspension was tilted so just the pellet with bacteria was left in the tube and 1,5ml 0,1M NaOH were added to dissolve the pellet. Then 1ml (1000µl) of this solution was putted into a cuvette and the OD was measured at 600 nm in a Hitatchi U-1100 Spectrophotometer. In the next challenge experiment one could now compare the OD in lye with the ones already done.

2.7 Freezing down bacteria

In order to ensure that there were enough bacteria for future studies I cultivated bacterial-suspension for freezing in Cryo tubes (à 1,8ml) at -80°C. First a preculture was made, in the exact same way as for the growth curves, which was incubated at 20°C for 5 days (or 3 days for P.g wt and P.g Pda8). Then 2% volume:- volume of the preculture was transferred into a new 250ml Erlenmeyer bulb with 20ml Marine Broth, this new culture was stored in the incubator until optimum OD was reached. The OD was measured at 600 nm in the Hitatchi U-1100 Spectrophotometer. After optimum OD was reached the culture was transferred to a 50ml blue centrifuge-tube with 4ml of autoclaved glycerol (added 20% glycerol to 80% bacterial suspension). The new suspension was well mixed, before 1ml was distributed to each of the 6 Cryo tubes, à 1,8ml. The tubes were named and marked with date before they were stored at -80°C.

2.8 80% aerated sterile seawater

In these challenge experiments, 80% aerated sterile seawater was used. Seawater was brought to the laboratory in several 5 litre bulbs from the Elab inflow of seawater. At the laboratory 20% of the seawater was replaced with distilled water before filtering the water trough a vacuum filter (Sarstedt No.83.1822, Filtropur V25 0,45 250ml). Then the 80% seawater was autoclaved (TOMY SX-700E) for 15 minutes. Before the water was distributed into the wells it was aerated to full gas saturation. We used 80% aerated sterile seawater (80% seawater, 20% distilled freshwater) because one would get precipitation of NaCl, salt-crystals, with regular (100%) seawater. Such 80% autoclaved aerated sterile seawater (28‰) or diluted seawater until 30‰ has been commonly used in other similar experiment (Hjelm et al., 2004, Sandlund and Bergh, 2008, Sandlund et al., 2010).

2.9 Challenge dose

The *Vibrio anguillarum* HI610 challenge dose were made by Kristian Dam, Department of Biology, University of Bergen, Norway. (More information about the procedure in Appendix 7.3)

To prepare the probiotic challenge doses one started with stopping the cultures as close to optimum OD as possible. Then a proper washing procedure had to be carried out, in order to avoid excess bacterial growth. The washing procedure started with transferring a proper amount (2x20ml) of probiotic culture from the 250ml Erlenmeyer bulbs to a 50ml sterile centrifuge tubes. Then the culture was centrifuged at 1500rpm in 8 minutes at 10°C in an Allegra X-15R Centrifuge (Beckman Coulter). The supernatant was poured out and the pellet was well mixed with the same amount, 80% autoclaved sterile seawater, as there was culture. Then the suspension was centrifuged a second time in the centrifuge at 1500rpm in 8 minutes at 7°C in an Allegra X-15R Centrifuge (Beckman Coulter) (12°C if the challenge dose are for 13°C or 15°C). The supernatant was poured out and the pellet was well mixed with the same amount, 80% autoclaved sterile seawater, as there was culture. After washing, the approximate number of bacteria in the suspension and the challenge dose had to be estimated. I tried to estimate the number of bacteria in the suspension by looking in the microscope and use the Tiefe counting chamber. This number had too high uncertainty, therefore a standardized procedure were worked out were OD in lye, NaOH, and CFU, plate-count were

used to set the concentration for the challenge doses. In all the challenge experiments there were used a 1:10 dilution of the main growth culture as challenge dose. See table 3.2 a) until 3.2 c) under Results for the challenge dose concentrations.

2.10 Challenge experiment

These challenge experiments were based on earlier studies from Bergh (2000) with some modifications. In the present challenge experiments, we tested different probiotic strains alone and together with the pathogen *Vibrio anguillarum* HI610 at 7°C and 13°C (15°C).

2.10.1 Standardised challenge experiment

The standardised method for each experiment was as follows: When the eggs arrived at Høyteknologisenteret in Bergen they were randomly selected and placed separately in wells in a 24-wells polystyrene multi-dish (Nunc, Roskilde, Denmark), each well with one egg is seen as an independent unit. Before the eggs arrived the wells were filled with 2 ml of 80% aerated sterile seawater. After the eggs were placed in the wells, the wells were challenged with *V. anguillarum* strain HI610 and/or the probiotic strains according to the protocol of the different challenge experiments. To each well 100µl of each used bacterial suspensions were added. For negative control 100µl of 80% aerated sterile seawater was added instead of bacterial suspension. For each experiments setup see table 7.6 a) – 7.6 c) in Appendix 7.6. The ideal final concentration of each bacterium in the wells were set to be "high dose", i,e. approximately 10^6 bacteria ml^{-1} based on total cell count, CFU or estimates by counting chamber technique. For the probiotics the final concentration in the well were a bit complicated to estimate, because of the uncertainty around CFU, but an approximately concentration are presented in table 3.2 c) under Results. These challenge experiments included one negative control group consisting of unchallenged larvae, one positive control group challenged with high dose, 10^6 CFU ml^{-1} *V.anguillarum* strain HI610, groups challenged with the different probiotics and groups challenged with the different probiotics and HI610 at the same time, probiotics 48hours before HI610 and HI610 48hours before the probiotic. It was used three plates for each challenge group. This gave a total of 72 larvae for each 24 treatment group to register mortality. The mortality was registered every day. The day

hatching reached 50%, was defined as day 0. Each group of three plates were placed on top of each other, and held at the same position throughout the experiment.

The eggs/larvae were kept in dark in a cooling room that kept the temperature 7°C +/- 1°C from the challenge day and for 14 days after hatching. We measured the temperature in the air conditioned room throughout the experiment and for 5 days, 2 times per day, before the experiment started to see that there was stabile temperature. The eggs/larvae were only disposed for light for a short time when we registered mortality.

Also a parallel challenge experiments at 13°C +/- 1°C (15°C) was carried out with the same procedures, but some differences were done because of the difficulties with the temperature increase of the water.

After finishing the 7°C challenge we transferred the eggs to 1liter beakers with 34‰ sterile seawater and oxygenating. Then the beakers with eggs were putted in an incubator for interval increase of the temperature from 7°C to 13°C, over a period of 24hours. After 24hours we used a climate room at 12°C were we randomly selected and placed the eggs separately in wells in a 24-wells polystyrene multi-dish (Nunc, Roskilde, Denmark) and carried out the same procedure as at the 7°C experiments. The challenged groups were put 6 and 6 on top of each other in an incubator (infors HT Minitron) set for 13°C. The day hatching reached 50%, was defined as day 0 and the mortality was registered every day like the 7°C experiments. The 13°C experiments I only had going for 10 days post hatch and not for 14 days like the 7°C experiments, as development of the poikilothermic larvae is faster at higher temperatures.

2.10.2 Challenge experiment number one

The set up for challenge experiment number one is to be found in table 7.6 a) in Appendix 7.6

The first experiment was carried out at one temperature only, 7°C and the eggs were delivered from hatchery A. In this challenge experiment there was a negative control group, and in addition to the high dose 10^6 CFU ml^{-1} *V. anguillarum* HI610 control group, a control group that were challenged with low dose 10^4 CFU ml^{-1} *V. anguillarum* HI610. There were also several groups challenged with different (high dose 10^6 CFU ml^{-1}) high doses of the different probiotics, high doses of the different probiotics and low dose of 10^4 CFU ml^{-1} *V. anguillarum* HI610, and groups with the different probiotics added 48hours before the low dose of 10^4 CFU ml^{-1} *V. anguillarum* HI610 was added.

2.10.3 Challenge experiment, number two

The set up for challenge experiment number two is to be found in table 7.6 b) in Appendix 7.6 Some setup changes from experiment number one to number two. One change was the parallel to 7°C at 15°C, and another change was that the eggs that were used were delivered from hatchery B. In these two parallels only two probiotics were used, *Phaeobacter* strain 27-4 and *Phaeobacter* strain M23-3.1, and a high challenge dose, 10^6 CFU ml^{-1}, with *Vibrio anguillarum* strain HI610. In addition there were a challenge group where high dose *V. anguillarum* HI610 were added 48hours before the probiotics were added. To do the 15°C experiment the temperature in the seawater with eggs were increased over two days, 48hours. The increase in temperature was achieved by transferring the transport water and eggs over in 1liter beakers with aerating. The beakers were then stored in an incubator were the temperature were gradually increased in intervals from 7°C to 15°C over 48hours. After 48hours when the eggs and water had reached 15°C we randomly selected and placed the eggs separately in wells before the different challenge doses were added. The distributions of the eggs were done in an air-conditioned room, set to 15°C. These parallels were ended 10 days post day 0.

2.10.4 Challenge experiment, number three

The set up for challenge experiment number three is to be found in table 7.6 c) in Appendix 7.6. In challenge experiment 3 there were in addition to *Phaeobacter* strain 27-4 and *Phaeobacter* strain M23-3.1 used two other probiotics, *Phaeobacter gallaeciensis* BS107-wt and the mutant *Phaeobacter gallaeciensis* BS107-Pda8, but with the same setup for challenge groups. The eggs were delivered from hatchery A. This time, the eggs were suppose to arrive closer to hatching than the earlier experiments, which resulted in shorter time to increase the temperature from 7°C to 15°C. The increase in temperature was done gradually over 24hours.

2.10.5 Challenge experiment number four and five

The set up for challenge experiment number four and five are to be found in table 7.6 c) in Appendix 7.6.

Challenge experiment 4 and 5 were done exactly as the standardized challenge experiment, described first in this section under 2.10.1 Standardised challenge experiment. The eggs used in challenge experiment number four were delivered from hatchery A, and the eggs used in challenge experiment number five were delivered from hatchery B.

2.11 Statistical analyses of mortality rates

Pairs of treatment groups were tested against each other to elucidate differences in mortality between the larval groups challenged with probiotics, the larval negative control group, the larval positive control group, the challenged larval groups at different temperatures and the challenged larval groups from the two different hatcheries. Group comparisons were planned before the experiment was carried out. Due to earlier studies (Sandlund et al., 2010) and the knowledge about when the yolk sac period ends and when starvation would have an impact (Holm, 1999, Kjørsvik et al., 1991) the multiple tests were performed at 10 days post hatch for the 7°C challenge experiments and at 8 days post hatch for the 13°C (and 15°C) challenge experiments. When the test were used to elucidate differences in mortality between the challenged larval groups at different temperature (7°C vs. 13°C) I chose to test the difference at 8 days post hatch for both groups. For each pair of treatment groups that was tested against each other I first created a frequency table describing the number of dead and alive larvae in the two groups. The H_0 that there is no difference in mortality between the two groups was then tested by using a Chi-square test for 2x2 contingency tables. These tests were performed using R, version 2.13.1 (R Foundation for Statistical Computing, Vienna, Austria) and the results are presented as "red marks" in the cumulative mortality graphs under Results, as a p-value in table 7.8 a) in Appendix 7.8 and as examples of matrix setup in Appendix 7.7.

2.12 Gram staining

In order to obtain pictures of the rosettes formed by the probiotic bacteria, Gram staining and fluorescent microscopy (ZEISS, Axioskop 2plus) was done.

A thin smear of broth culture, with growth around optimum OD, on a slide was made. The smear was air dried before it was heat-fixed. This was done by taking the slide, with the sample-side facing away from the flames, trough the flame a few times. The smear was flooded with Crystal Violet solution for 1 minute. Then it was briefly washed with water and all the excess water was drained off. The sample-side was facing down from the water flow. Once again the smear was flooded, this time with Iodine solution for 1 minute, before it was washed off with water. The smear was destained with ethanol until no more colour flows off

the smear. Before the smear was counter-strained with Safranin for 10 seconds, the ethanol was washed off. The smear was washed in water one last time, and then air-dried before a cover-slip was fastened over the sample area so one could examine the smear under oil-immersion. Then I looked at the stained material in a fluorescent microscopy (ZEISS, Axioskop 2plus) with bright-field illumination and took pictures. Gram (-) bacteria stains red and Gram (+) bacteria stains blue. Mine bacteria were mainly Gram (-) and stained red. Pictures of some of the different rosettes making probiotics are showed in the Results section in figure 3.5.

3 Results

3.1 Growth curves

Growth curves for the different bacteria used in the challenge experiments are shown in the following figures 3.1 a) – 3.1 i). All the growth curves have a slope upwards, with exponential growth, before the bacteria goes into stationary phase. For the bacteria where I managed to grow CFU, Colony Forming Units on Marine agar -plates, the CFU logarithms are plotted slightly higher than the measured OD. From these curves one decided where and when the optimum OD would appear, and used this information to take out/stop the challenge dose when the bacteria cultures were around optimal growth. In general the maximum growth rate for the *Vibrio anguillarum* HI610 was achieved after 300-360 min (Fig. 3.1 a)), whereas for the probiotics (Figures 3.1 b) -3.1 i)), growth was slower.

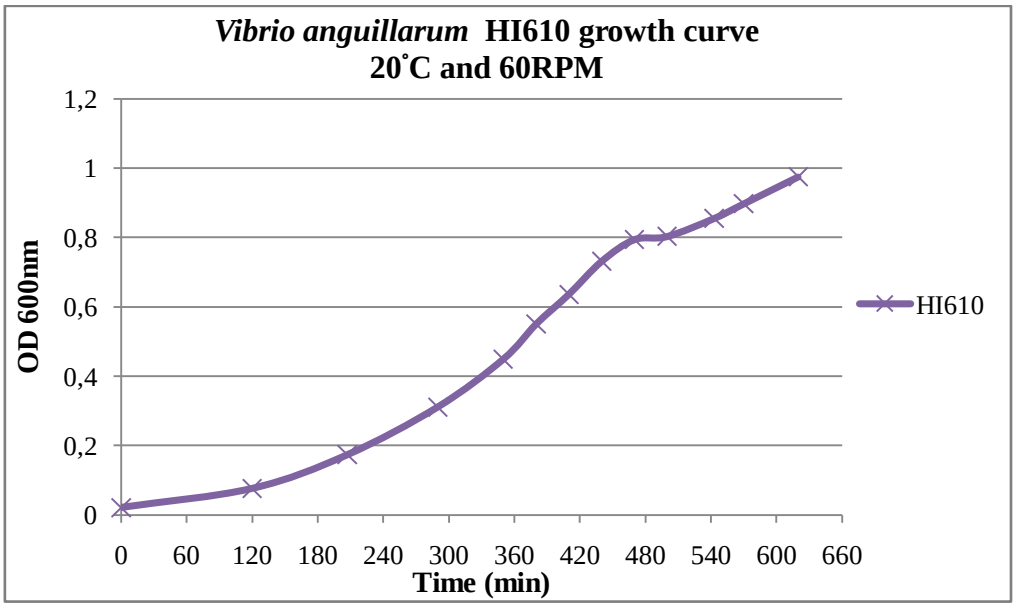

Figure 3.1 a): Growth over time in a *Vibrio anguillarum* strain HI610 and Tryptic Soy Broth culture, measured at optical density, OD, at 600nm in a Hitatchi U-1100 Spectrophotometer. Carried out by Kristian Dam, Department of Biology, University of Bergen, Norway.

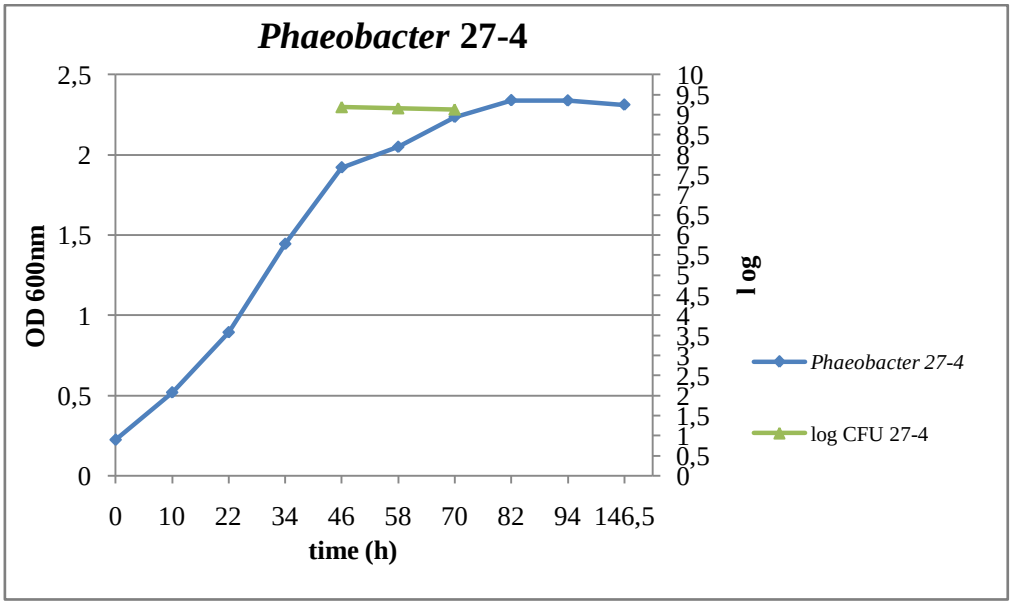

Figure 3.1 b): Growth over time in a *Phaeobacter* strain 27-4 and Marine Broth culture measured at optical density, OD, at 600nm in a Hitatchi U-1100 Spectrophotometer. After 46h, 58h and 70h there were also made CFU-plate estimate.

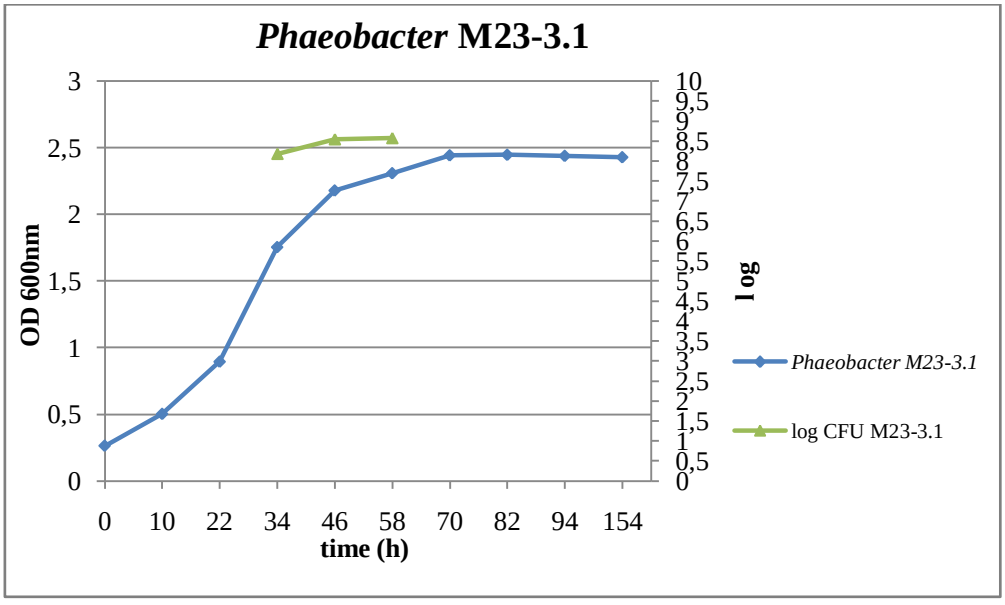

Figure 3.1 c): Growth over time in a *Phaeobacter* strain M23-3.1 and Marine Broth culture measured at optical density, OD, at 600nm in a Hitatchi U-1100 Spectrophotometer. After 34h, 46h and 58h there were also made CFU-plate estimate.

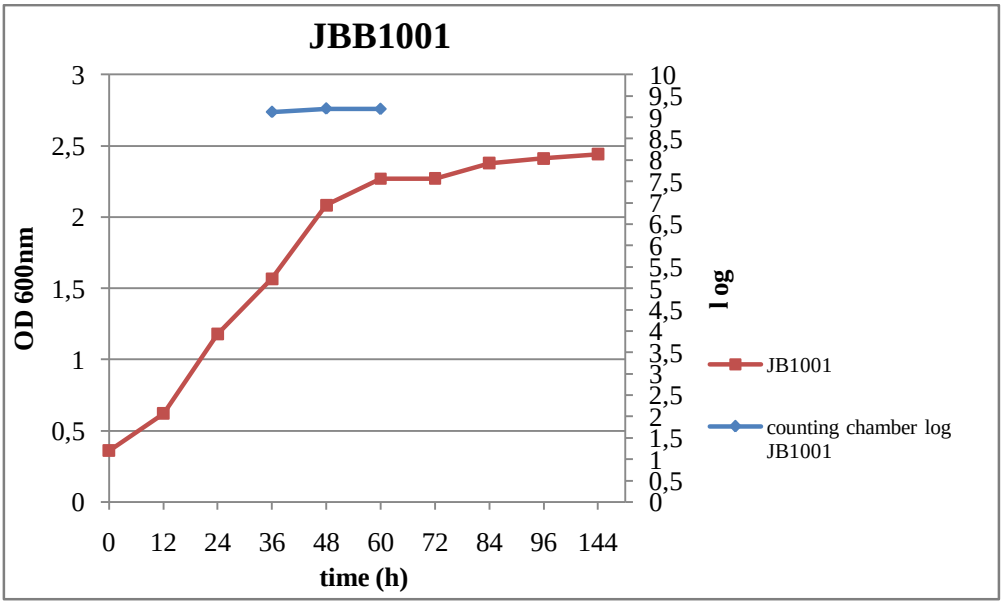

Figure 3.1 d): Growth over time in a JBB1001 (mutant) and Marine Broth (with kanamycin) culture measured at optical density, OD, at 600nm in a Hitatchi U-1100 Spectrophotometer. After 36h, 48h and 60h there were also made a counting chamber estimate. The CFU-plate was inappropriate.

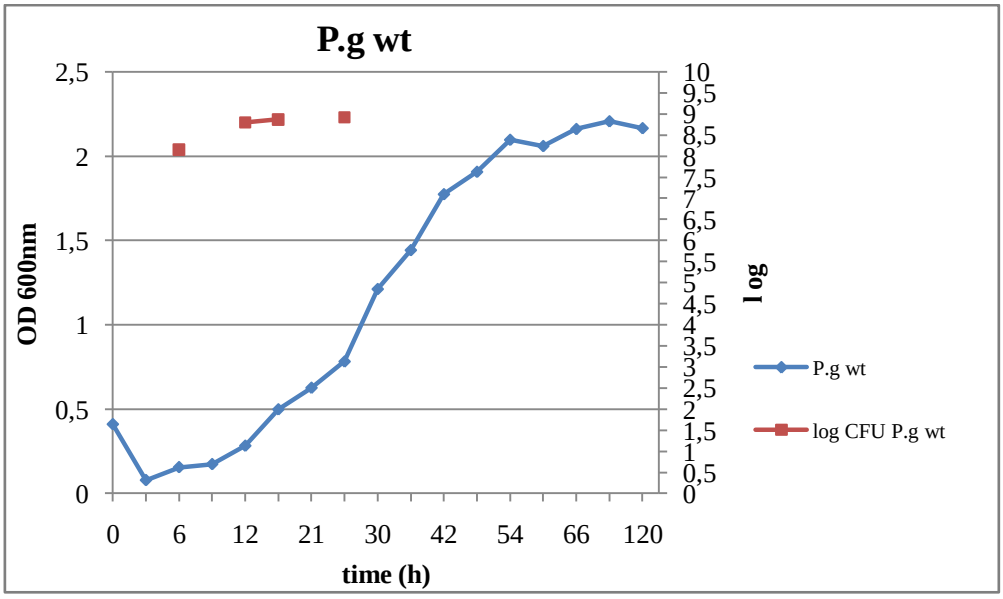

Figure 3.1 e): Growth over time in a *Phaeobacter gallaeciensis* BS107-wt and Marine Broth culture measured at optical density, OD, at 600nm in a Hitatchi U-1100 Spectrophotometer. After 6h, 12h, 18h and 24h there were also made CFU-plate estimates.

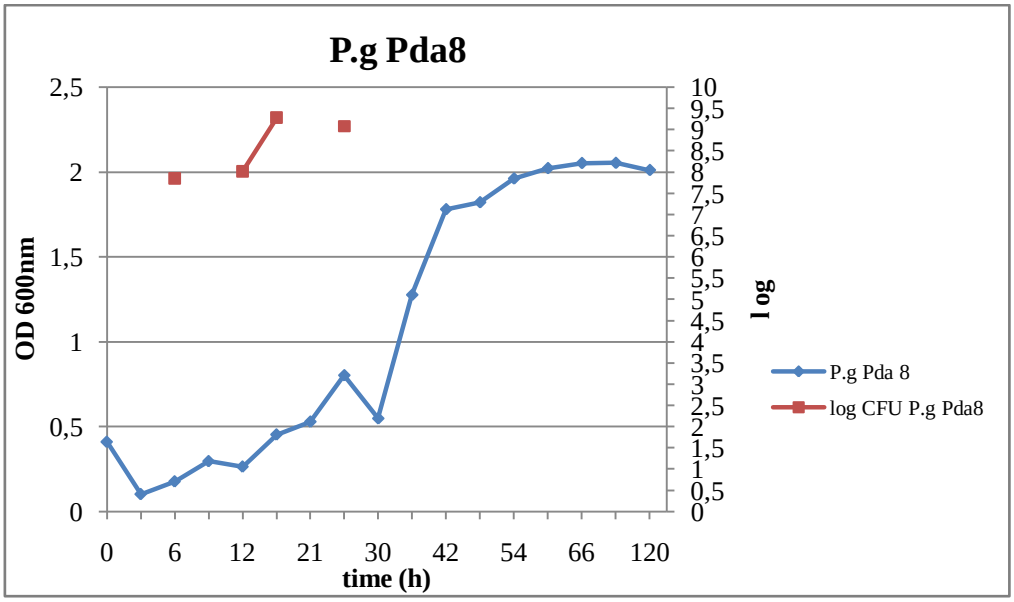

Figure 3.1 f): Growth over time in a *Phaeobacter gallaeciensis* BS107-Pda8 and Marine Broth culture measured at optical density, OD, at 600nm in a Hitatchi U-1100 Spectrophotometer. After 6h, 12h, 18h and 24h there were also made CFU-plate estimates.

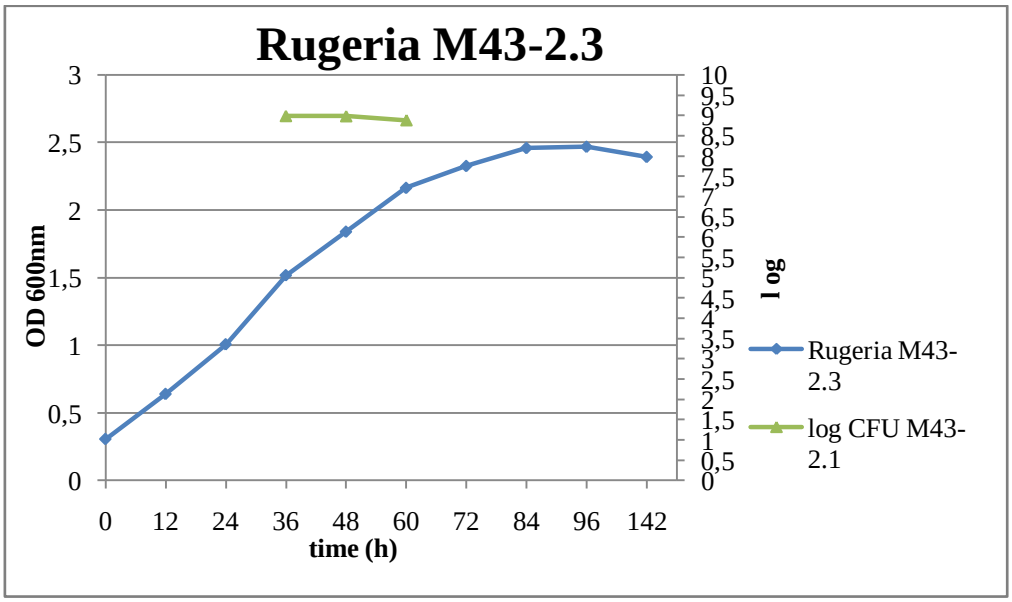

Figure 3.1 g): Growth over time in a *Rugeria* strain M43-2.3 and Marine Broth culture measured at optical density, OD, at 600nm in a Hitatchi U-1100 Spectrophotometer. After 36h, 48h and 60h there were also made CFU-plate estimates.

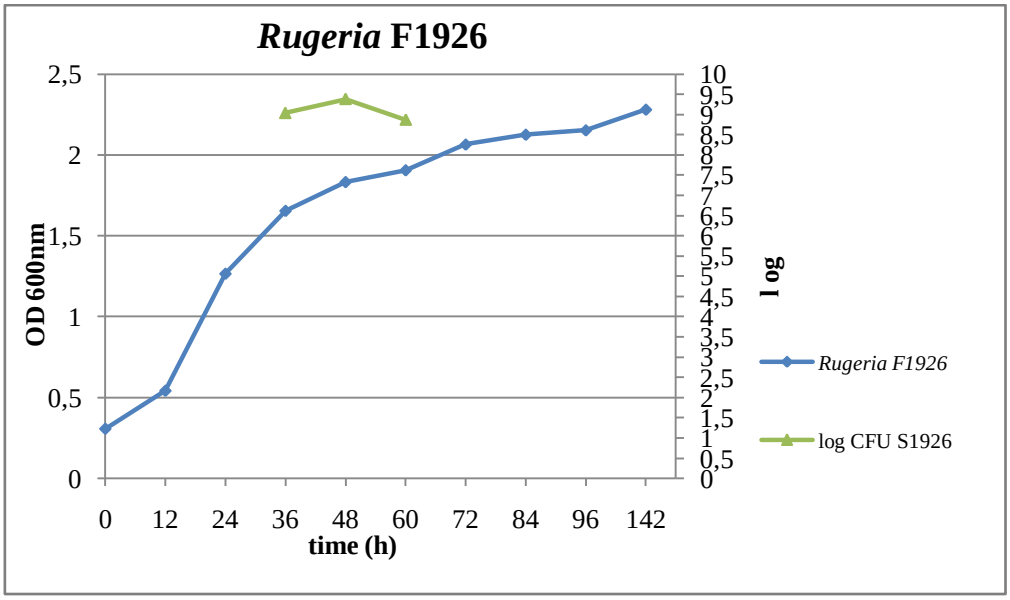

Figure 3.1 h): Growth over time in a *Rugeria* strain F1926 and Marine Broth culture measured at optical density, OD, at 600nm in a Hitatchi U-1100 Spectrophotometer. After 36h, 48h and 60h there were also made CFU-plate estimates.

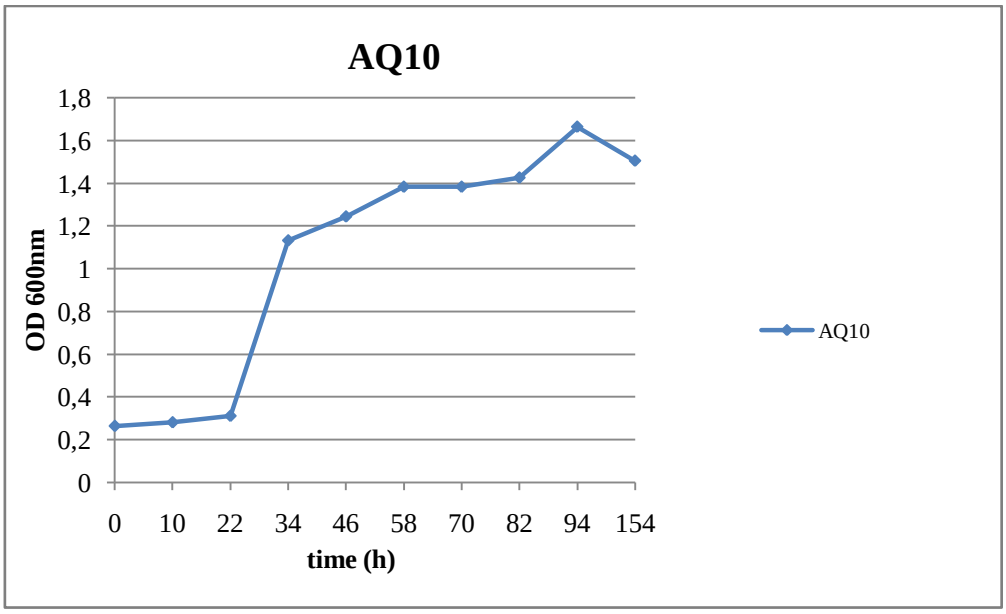

Figure 3.1 i): Growth over time for a *Pseudoalteromonas citrea* strain AQ10 and Marine Broth culture measured at optical density, OD, at 600nm in a Hitatchi U-1100 Spectrophotometer. The bacteria were too small to count in the microscope, thus OD was used.

3.2 Challenge dose

The *Vibrio anguillarum* HI610 challenge dose were made by Kristian Dam, Department of Biology, University of Bergen, Norway. Except from in challenge experiment number one where I used both high (10^6 CFU ml^{-1}) and low (10^4 CFU ml^{-1}) dose *V.anguillarum* HI610, I only used high dose (10^6 CFU ml^{-1}) in the rest of the challenge experiments 2, 3, 4 and 5.

Table 3.2 a): Challenge doses, challenge experiment one. In challenge experiment number one, challenge doses were made for one challenge (one point on the time scale) and the measured optical densities, OD, at 600nm for the washed suspensions is given:

washed bacteria suspension	optical density, OD
Phaeobacter 27-4	0,183
Phaeobacter M23-3.1	0,216
Rugeria F1926	0,181
Rugeria M43-2.1	0,185

Table 3.2 b): Challenge doses, challenge experiment two. In challenge experiment number two I used a gentler washing procedure than in the first challenge experiment, and there were two challenges for each temperature with a time laps between them at 48 hours. The optical densities, OD, for the washed suspensions at 600nm and the OD in NaOH at 360nm in the 1:10 dilution are given:

washed bacteria suspension	optical density, OD	OD in NaOH (in 1:10 dilution)
first challenge dose for 7°C:		
Phaeobacter 27-4	1,000	0,064
Phaeobacter M23-3.1	1,226	0,076
second (48hours after) challenge dose 7°C and first challenge dose for 15°C:		
Phaeobacter 27-4	1,051	0,066
Phaeobacter M23-3.1	1,387	0,069
second (48hours after) challenge dose 15°C:		
Phaeobacter 27-4	1,051	0,066
Phaeobacter M23-3.1	1,387	0,069

Table 3.2 c): Challenge doses, challenge experiment 3-5. Table of measured OD in start culture, OD in NaOH in the 1:10 dilution, concentration or CFU in the 1:10 dilution (challenge dose), log CFU and final concentration in well for all the challenge doses in challenge experiments 3, 4 and 5.

experiment no.	Probiont	OD in start culture	OD in NaOH (in 1:10 dilution)	concentration in 1:10 dilution (the challenge dose)	log CFU	final concentration in well
3 day 1	27-4	1,833	0,115	7,83E+08	8,89	3,92E+07
7°C	M23-3.1	2,244	0,190	1,20E+10	10,08	6,00E+08
	P.g wt	1,864	0,178	8,00E+07	7,90	4,00E+06
	P.g Pda8	1,894	0,162	2,63E+09	9,42	1,32E+08
3 day 2	27-4	1,658	0,114	3,69E+09	9,57	1,85E+08
15°C	M23-3.1	2,155	0,167	1,20E+08	8,08	6,00E+06
	P.g wt	1,838	0,186	8,67E+08	8,94	4,34E+07
	P.g Pda8	1,731	0,223	3,70E+08	8,57	1,85E+07
3 day 3	27-4	1,809	0,114	1,04E+09	9,02	5,20E+07
7°C	M23-3.1	2,209	0,188	9,67E+07	7,99	4,84E+06
	P.g wt	1,982	0,225	-	-	-
	P.g Pda8	1,811	0,289	7,20E+08	8,86	3,60E+07
3 day 4	27-4	2,252	0,148	8,00E+09	9,90	4,00E+08
15°C	M23-3.1	2,310	0,238	4,67E+07	7,67	2,34E+06
	P.g wt	2,220	0,251	2,40E+08	8,38	1,20E+07
	P.g Pda8	2,177	0,342	2,76E+09	9,44	1,38E+08

4 day 1	27-4	2,219	0,132	1,11E+08	8,05	5,55E+06
7°C	M23-3.1	2,300	0,217	8,67E+07	7,94	4,34E+06
	P.g wt	2,122	0,176	6,97E+08	8,84	3,49E+07
	P.g Pda8	1,911	0,236	1,00E+10	10,00	5,00E+08
4 day 2	27-4	2,230	0,145	7,20E+08	8,86	3,60E+07
13°C	M23-3.1	2,307	0,158	1,83E+08	8,26	9,15E+06
	P.g wt	2,145	0,212	4,08E+09	9,61	2,04E+08
	P.g Pda8	1,848	0,253	-	-	-
4 day 3	27-4	2,179	0,115	-	-	-
7°C	M23-3.1	2,189	0,232	1,00E+08	8,00	5,00E+06
	P.g wt	2,068	0,207	8,35E+08	8,92	4,18E+07
	P.g Pda8	1,920	0,254	3,50E+08	8,54	1,75E+07
4 day 4	27-4	2,278	0,130	3,85E+09	9,59	1,93E+08
13°C	M23-3.1	2,269	0,200	8,67E+07	7,94	4,34E+06
	P.g wt	2,222	0,208	1,07E+08	8,03	5,35E+06
	P.g Pda8	2,160	0,270	4,57E+08	8,66	2,29E+07
5 day 1	27-4	2,290	0,131	4,00E+09	9,60	2,00E+08
7°C	M23-3.1	2,316	0,151	1,47E+08	8,17	7,35E+06
	P.g wt	2,211	0,250	1,03E+09	9,01	5,15E+07
	P.g Pda8	1,976	0,242	3,12E+09	9,49	1,56E+08
5 day 2	27-4	2,113	0,111	-	-	-
13°C	M23-3.1	2,294	0,168	3,77E+08	8,58	1,89E+07
	P.g wt	2,226	0,225	2,61E+09	9,42	1,31E+08
	P.g Pda8	1,998	0,271	1,31E+09	9,12	6,55E+07
5 day 3	27-4	2,051	0,141	-	-	-
7°C	M23-3.1	2,184	0,204	4,04E+09	9,61	2,02E+08
	P.g wt	2,085	0,231	5,49E+09	9,74	2,75E+08
	P.g Pda8	1,837	0,264	8,00E+09	9,90	4,00E+08
5 day 4	27-4	2,037	0,106	2,60E+08	8,41	1,30E+07
13°C	M23-3.1	2,459	0,251	1,57E+08	8,20	7,85E+06
	P.g wt	2,398	0,228	1,26E+09	9,10	6,30E+07
	P.g Pda8	2,295	0,280	-	-	-

3.3 Hatching

3.3.1 Challenge experiment number one

Six days (144 hours) after arrival and transferred into wells there were over 50% hatching. This day was defined as day 0. A total of 75% of the eggs hatched successfully.

3.3.2. Challenge experiment number two

In challenge experiment number two there was a parallel to the one at 7°C, at 15°C. In the parallel at 7°C the eggs had 50% hatching, or more precisely 60,4%, seven days (approximately 168 hours) after arrival and transferred into wells. This day was considered as day 0. A total of 86,5% of the eggs hatched successfully at 7°C. In the 15°C parallel, hatching was around 50% five days (120 hours) after arrival and transferred into wells. This day was defined as day 0. A total of 80% of the eggs hatched successfully.

3.3.3. Challenge experiment number three

Seven days (168 hours) after arrival and transferred into wells there were over 50% hatching, approximately 81% hatching, in the 7°C parallel. This day was defined as day 0. The total of eggs that hatched successfully was around 89,9%.

In the 15°C parallel the hatching never reached day 0 as defined by 50% hatching. The total of eggs that hatched successfully was 34%.

3.3.4. Challenge experiment number four

Seven days (168 hours) after arrival and transferred into wells there were over 50% hatching in the 7°C parallel. This day was defined as day 0. A total of 86% of the eggs hatched successfully. In the 13°C parallel the hatching was around 50%, or more precisely 79,7%, six days (144 hours) after arrival and transferred into wells. This day was then defined as day 0. A total of 85,4% of the eggs hatched successfully.

3.3.5. Challenge experiment number five

The eggs in challenge experiment number five were delivered closer to hatching, and while we were working a few eggs hatched. Three days (72 hours) after arrival and transferred into wells there were over 50% hatching, as much as 93,7% of the eggs were hatched, in the 7°C

parallel. At day two (48 hours) there were only 21,4% hatching. Day three (72 hours) was defined as day 0. A total of 94% of the eggs hatched successfully. In the 13°C parallel the hatching were over 50% only two days after arrival and transferred into wells. This day was defined as day 0. A total of 88,5% of the eggs hatched successfully.

3.4 Cumulative mortality

The percentage of cumulative mortality was calculated and plotted against dph (days post hatch) for all the different challenge groups. See graphs in figures under part chapter 3.4.1 to 3.4.7. All 72 eggs in each group, also the once that did not hatch, are concluded in the cumulative mortality calculations. The mortality was registered for 10, 12 or 14 days post hatch. The groups challenged with high dose (10^6 CFU ml^{-1}) *V.anguillarum* HI610 showed a high and rapid mortality, reaching near 100% cumulative mortality, in all the challenge experiments. In the negative control groups and the groups were probiotics are the only bacteria added there is minimal mortality. The different probiotics that are used in combination with *V.anguillarum* HI610 showed a variety of mortality, in the grade they have probiotic effect. The challenge experiments at 13°C (15°C) showed overall a higher mortality and mortality at an earlier stage (in terms of time) after day 0.

3.4.1: Challenge experiment number one

From challenge experiment number one only some of the results are shown in figure 3.4.1. This is because the first challenge experiment was a preliminary experiment, where the method was tested. There was higher mortality in all the challenge groups in challenge experiment number one because of fewer successfully hatched eggs and some wells were missing eggs. This is shown in the graph under figure 3.4.1, with more mortality at day 0. The graph shows high mortality in the high dose positive control group, and lower mortality in all the other groups. The results for this challenge experiment could only be used to tell that the probiotics did not have any negative or lethal effect on the larvae. No further statistical calculations were done for this experiment.

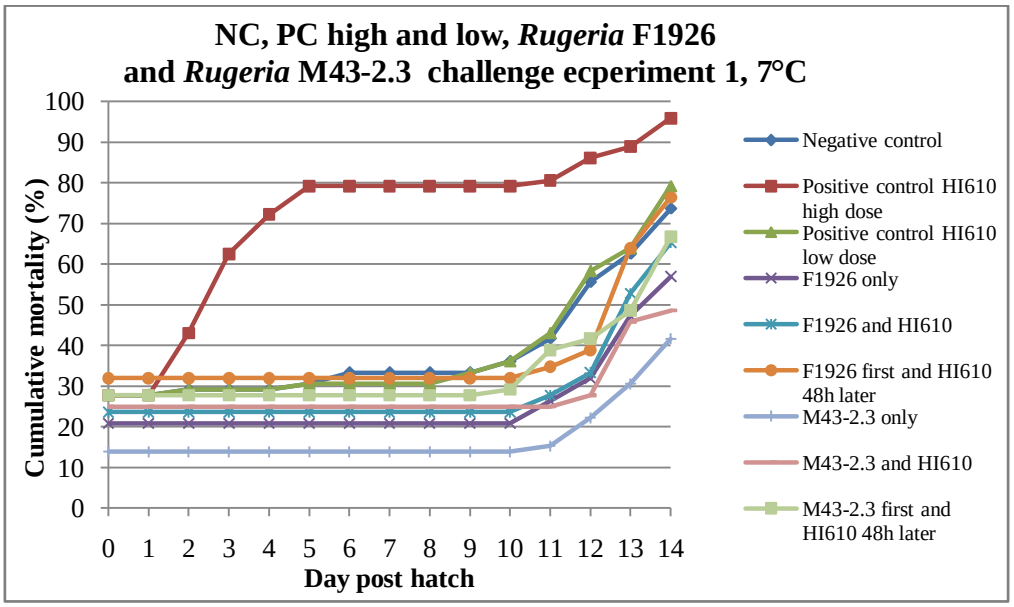

Figure 3.4.1: Percentage of cumulative mortality of non-feeding cod larvae challenged with *Ruegeria* F1926, *Ruegeria* M43-2.3 and *V.anguillarum* HI610, alone, one probiotic and the pathogen together at the same time and with a time laps of 48hours. The challenge doses are approximately high doses (10^6 CFU ml^{-1}) and one low dose (10^6 CFU ml^{-1}) PC, positive control. NC, negative control has no challenge dose added. Day 0: day of hatching.

3.4.2: Challenge experiment number two

In challenge experiment number two all the different challenge groups are included in one graph for the 7°C experiment, figure 3.4.2 a) and one graph for the 15°C experiment, figure 3.4.2 b). The cumulative mortality is registered from day 0 to 10 days post hatch. The cumulative mortality in the 7°C experiment is high and rapid mortality in positive control, little mortality in negative control, little mortality in challenge groups with only probiotic and in the challenge groups were the probiotic was added 48 hours before the pathogen. The challenge groups where *V.anguillarum* HI610 was added 48 hours before the probiotic, had high mortality for the *Phaeobacter* 27-4, but for the challenge group with *Phaeobacter* M23-3.1 there was minimal mortality. No statistical calculation was done for this experiment as the same challenge groups and the 7°C temperature are done with statistical calculations in challenge experiment number, 3, 4 and 5.

The cumulative mortality in the 15°C experiment shows an exponential cumulative mortality for all the challenge groups. No statistical calculations were done for this experiment, due to the fact that the graph indicates mortality for all larvae if several days with registration were done.

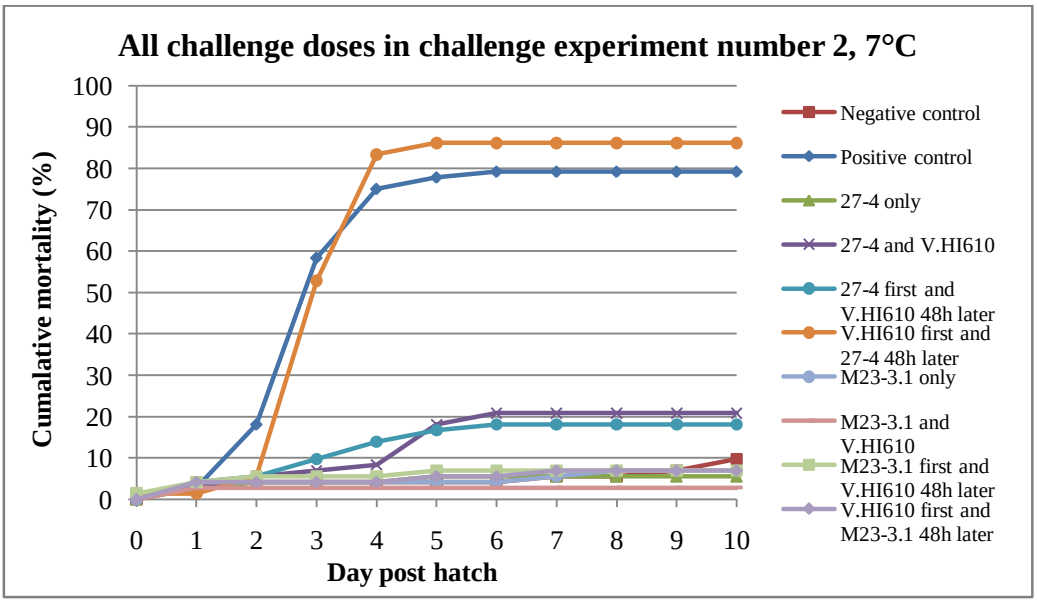

Figure 3.4.2 a): Percentage of cumulative mortality of non-feeding cod larvae challenged with *Phaeobacter* 27-4, *Phaeobacter* M23-3.1 and *V.anguillarum* HI610, alone, one probiotic and the pathogen together at the same time and with a time laps of 48hours. The challenge doses are approximately high doses (10^6 CFU ml^{-1}). Negative control has no challenge dose added. Day 0: day of hatching.

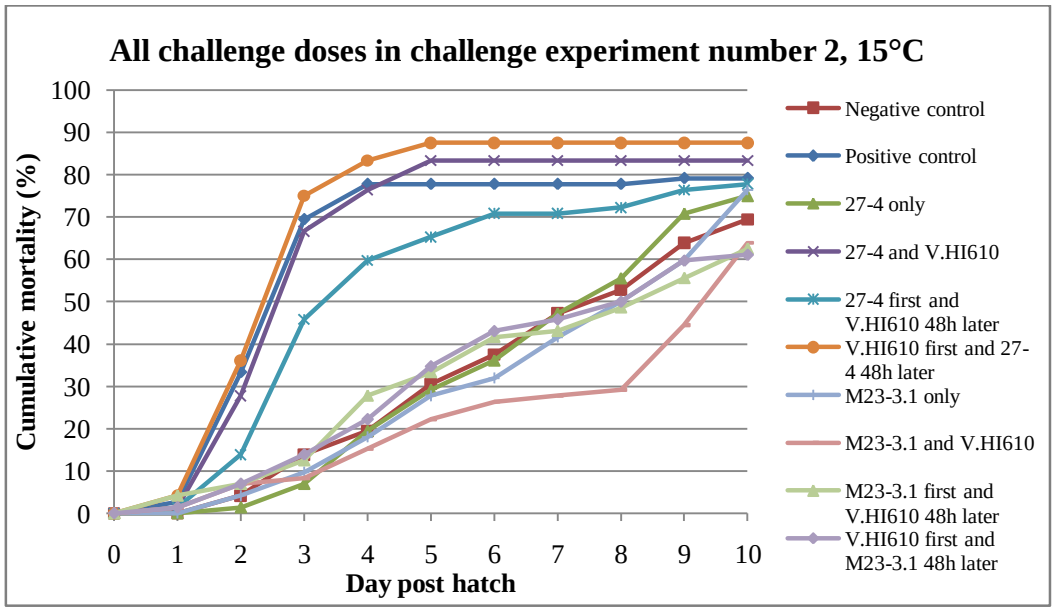

Figure 3.4.2 b): Percentage of cumulative mortality of non-feeding cod larvae challenged with *Phaeobacter* 27-4, *Phaeobacter* M23-3.1 and *V.anguillarum* HI610, alone, one probiotic and the pathogen together at the same time, and with a time laps of 48hours. The challenge doses are approximately high doses (10^6 CFU ml^{-1}). Negative control has no challenge dose added. Day 0: day of hatching.

3.4.3. Challenge experiment number three

The graphs for the 7°C challenge experiment are shown in figures 3.4.3 a) – 3.4.3 d).

In the 7°C parallel in challenge experiment number three the positive control show a significant higher mortality than all the other challenge groups tested, except from the "V.HI610 first and 27-4 48hrs after" group and the "V.HI610 first and P.g Pda8 48hrs after" group. The negative control group show some mortality (30 % 5dph) but were still significant ($p<<0{,}001$) different from the positive control group.

For the 15°C challenge experiment there are no results in graphs. The short time and leap for increase in temperature, in addition to incubator problems resulted in an earlier termination of the 15°C experiment and inconclusive results. There were only 34% hatching and the eggs that hatched died just few (2-3) days after hatching. The experiment was ended five days after the first registrations of hatching and lesser than 34% of the hatched larvae were alive.

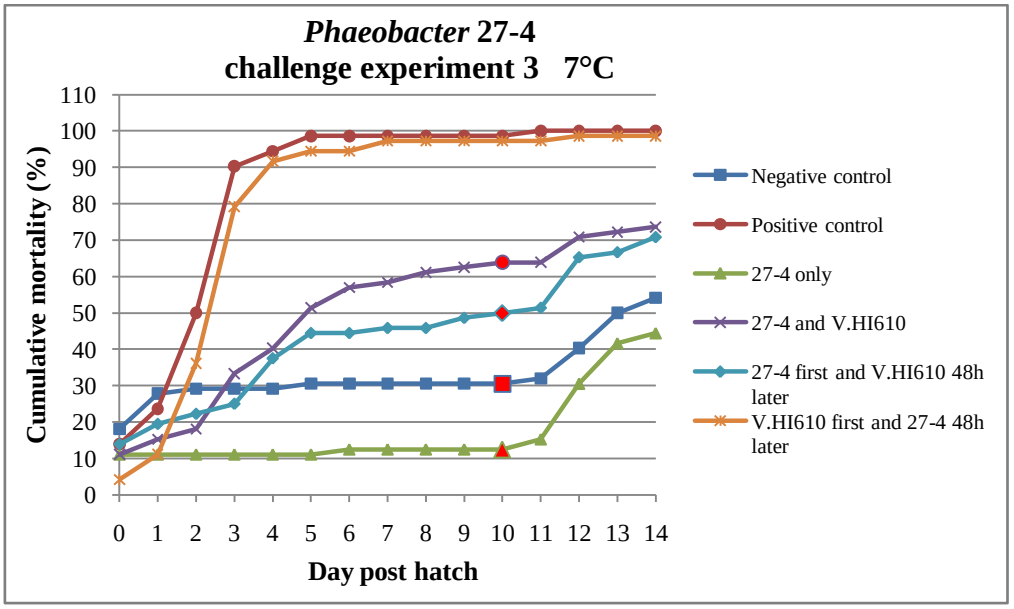

Figure 3.4.3 a): Percentage of cumulative mortality of non-feeding cod larvae challenged with *Phaeobacter* 27-4 and *V.anguillarum* HI610, alone, together at the same time and with a time laps of 48hours. All challenge doses are approximately high doses (10^6 CFU ml^{-1}). Negative control has no challenge dose added. Day 0: day of hatching. Red symbols (4): mortality rates 10dph significantly different from the positive control group, p-value (p<<0,001).

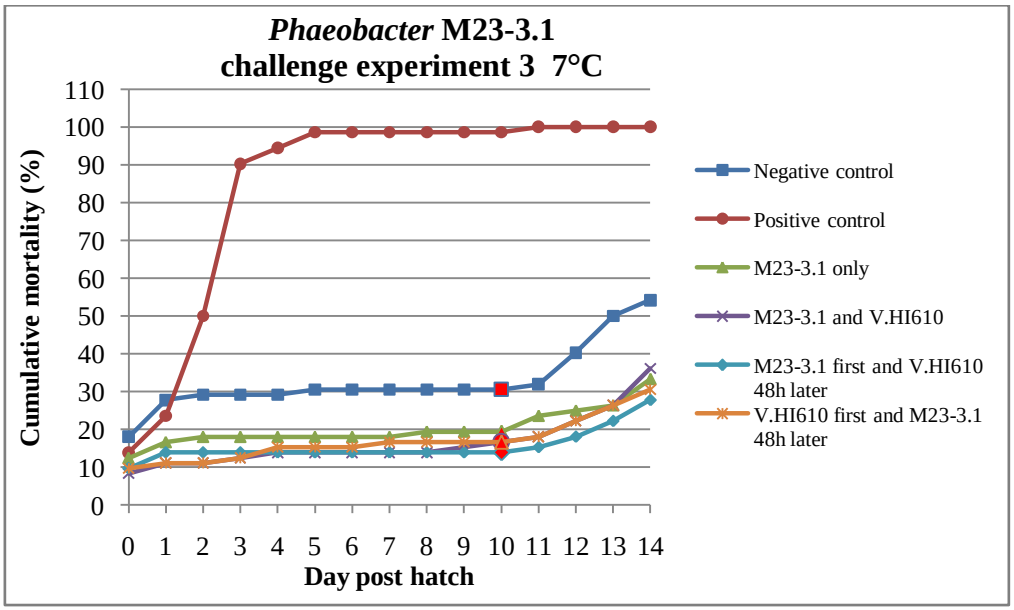

Figure 3.4.3 b): Percentage of cumulative mortality of non-feeding cod larvae challenged with *Phaeobacter* M23-3.1 and *V.anguillarum* HI610, alone, together at the same time and with a time laps of 48hours. All challenge doses are approximately high doses (10^6 CFU ml^{-1}). Negative control has no challenge dose added. Day 0: day of hatching. Red symbols (5): mortality rates 10dph significantly different from the positive control group, p-value (p<<0,001).

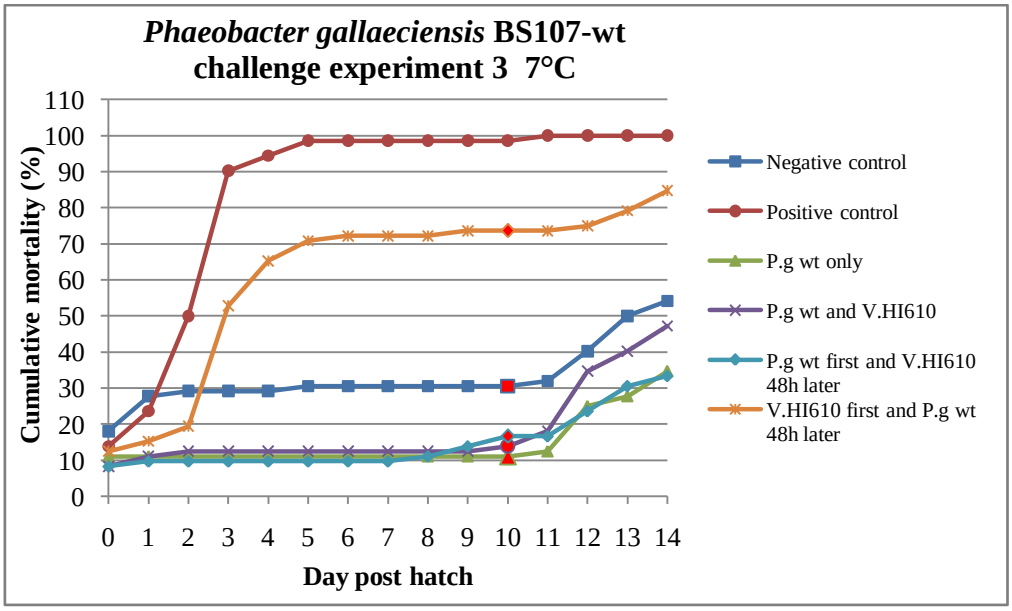

Figure 3.4.3 c): Percentage of cumulative mortality of non-feeding cod larvae challenged with *Phaeobacter gallaeciensis* BS107-wt and *V.anguillarum* HI610, alone, together at the same time and with a time laps of 48hours. All challenge doses are approximately high doses (10^6 CFU ml^{-1}). Negative control has no challenge dose added. Day 0: day of hatching. Red symbols (5): mortality rates 10dph significantly different from the positive control group, p-value ($p<<0,001$).

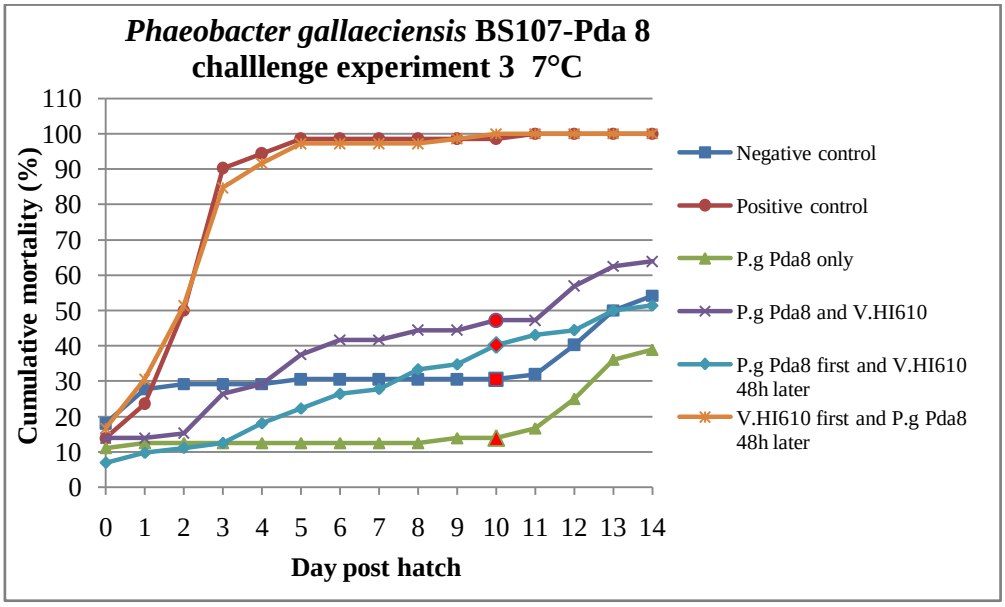

Figure 3.4.3 d): Percentage of cumulative mortality of non-feeding cod larvae challenged with *Phaeobacter gallaeciensis* BS107-Pgd8 and *V.anguillarum* HI610, alone, together at the same time and with a time laps of 48hours. All challenge doses are approximately high doses (10^6 CFU ml^{-1}). Negative control has no challenge dose added. Day 0: day of hatching. Red symbols (4): mortality rates 8dph significantly different from the positive control group, p-value ($p<<0,001$).

3.4.4. Challenge experiment number four

The graphs for challenge experiment number four at 7°C are shown in figures 3.4.4 a) – 3.4.4 d). The negative control group shows some mortality (over 40% 2dph) but are still significantly ($p<<0,001$) different from the positive control group mortality. The groups with only probiotics and the groups with probiotic added 48 hours before *V.anguillarum* HI610 shows little mortality and are significant ($p<<0,001$) different from the positive control group and some, especially the groups M23-3.1 only, P.g wt only and P.g Pda8 only have significantly lower mortality than the negative control group . In the groups were the probiotic is added at the same time as the pathogen there is differences in the cumulative mortality. *Phaeobacter* M23-3.1 and *V.anguillarum* HI610, and *Phaeobacter gallaeciensis* BS107-wt and *V.anguillarum* HI610 shows a lower mortality and are significant ($p<<0,001$) different from the positive control group, in contrast to *Phaeobacter* 27-4 and *V.anguillarum* HI610, and *Phaeobacter gallaeciensis* BS107-Pda8 and *V.anguillarum* HI610 which shows no significant ($p >1$) difference from the positive control group. In the groups were the *V.anguillarum* HI610 are added 48 hours before the probiotic the mortality is high and there is no significant ($p >1$) difference from the positive control group where the *V.anguillarum* HI610 is added 48hrs before the *Phaeobacter* 27-4 and the *Phaeobacter gallaeciensis* BS107-Pda8. For the groups were *V.anguillarum* HI610 are added 48 hrs before the *Phaeobacter* M23-3.1 and the *Phaeobacter gallaeciensis* BS107-wt, the mortality are significant lower than the mortality in the positive control group.

The graphs for challenge experiment number four at 13°C are shown in figures 3.4.4 e) – 3.4.4 h). In challenge experiment number four at 13°C all the challenge groups, except one were significant different from the positive control at 8dph. Some of the p-values were not that distinct, but even though significant. The challenge group that did not show significant difference from the positive control was the challenge group V.HI610 first and 27-4 48hrs after.

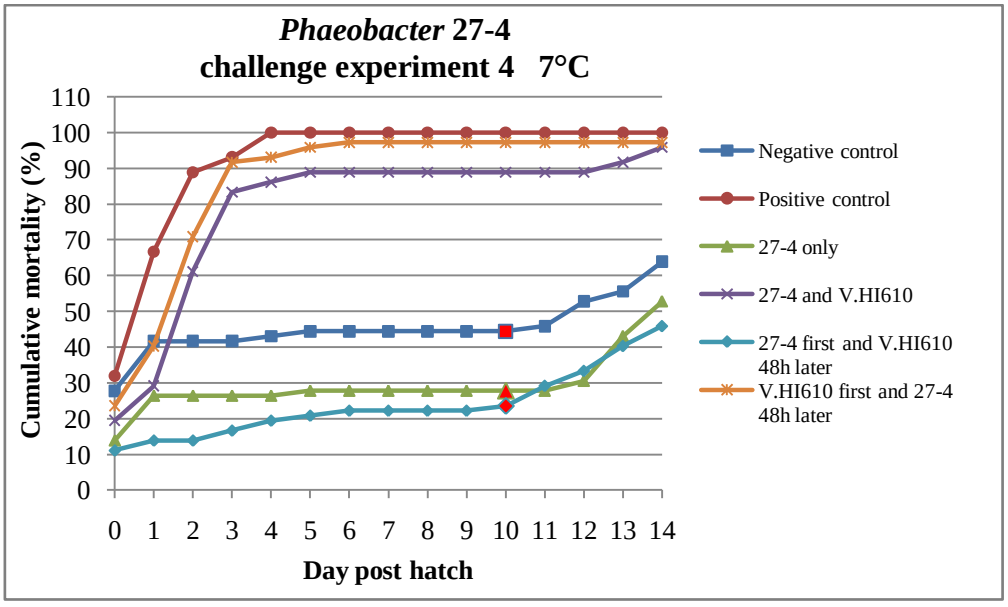

Figure 3.4.4 a): Percentage of cumulative mortality of non-feeding cod larvae challenged with *Phaeobacter* 27-4 and *V.anguillarum* HI610, alone, together at the same time and with a time laps of 48hours. All challenge doses are approximately high doses (10^6 CFU ml^{-1}). Negative control has no challenge dose added. Day 0: day of hatching. Red symbols (3): mortality rates 10dph significantly different from the positive control group, p-value (p<<0,001).

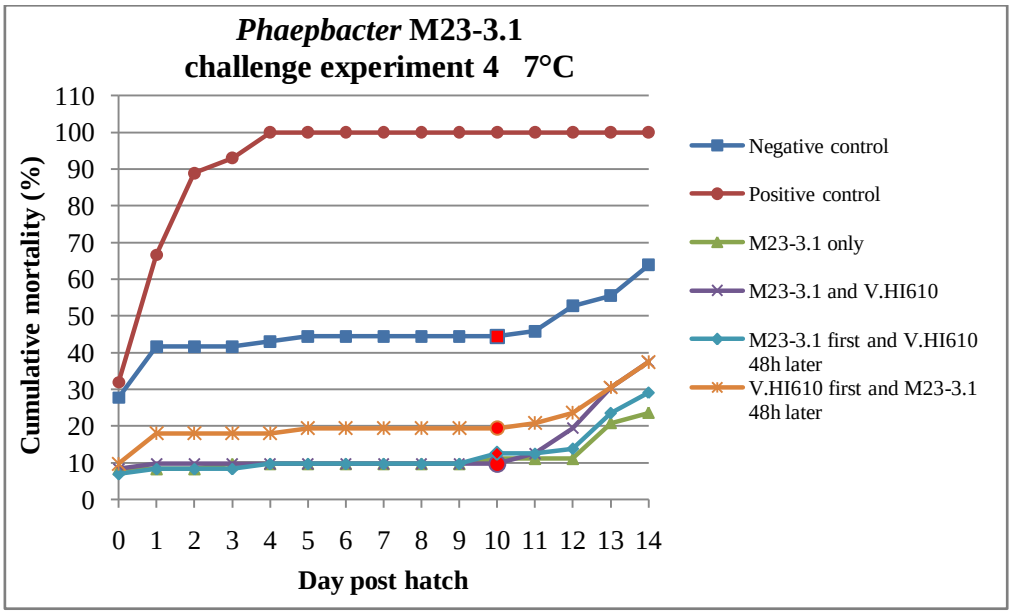

Figure 3.4.4 b): Percentage of cumulative mortality of non-feeding cod larvae challenged with *Phaeobacter* M23-3.1 and *V.anguillarum* HI610, alone, together at the same time and with a time laps of 48hours. All challenge doses are approximately high doses (10^6 CFU ml^{-1}). Negative control has no challenge dose added. Day 0: day of hatching. Red symbols (5): mortality rates 10dph significantly different from the positive control group, p-value (p<<0,001).

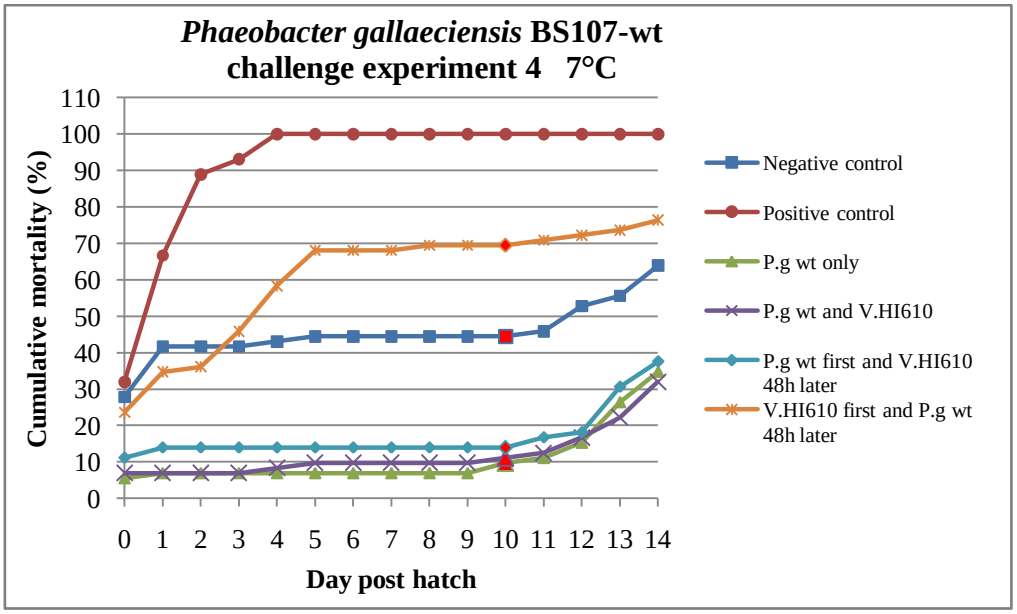

Figure 3.4.4 c): Percentage of cumulative mortality of non-feeding cod larvae challenged with *Phaeobacter gallaeciensis* BS107-wt and *V.anguillarum* HI610, alone, together at the same time and with a time laps of 48hours. All challenge doses are approximately high doses (10^6 CFU ml^{-1}). Negative control has no challenge dose added. Day 0: day of hatching. Red symbols (5): mortality rates 10dph significantly different from the positive control group, p-value (p<<0,001).

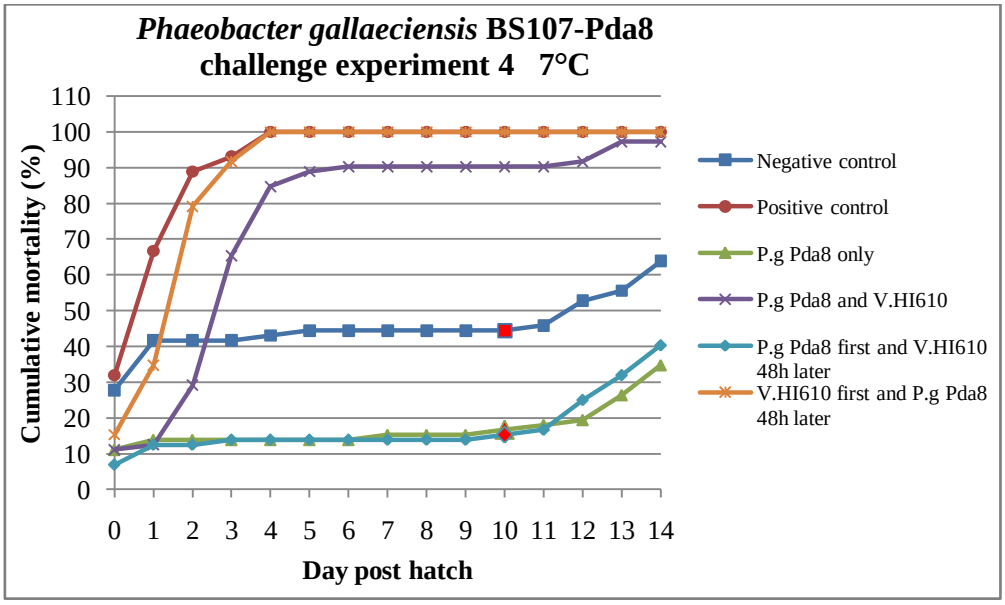

Figure 3.4.4 d): Percentage of cumulative mortality of non-feeding cod larvae challenged with *Phaeobacter gallaeciensis* BS107-Pgd8 and *V.anguillarum* HI610, alone, together at the same time and with a time laps of 48hours. All challenge doses are approximately high doses (10^6 CFU ml^{-1}). Negative control has no challenge dose added. Day 0: day of hatching. Red symbols (3): mortality rates 8dph significantly different from the positive control group, p-value (p<<0,001).

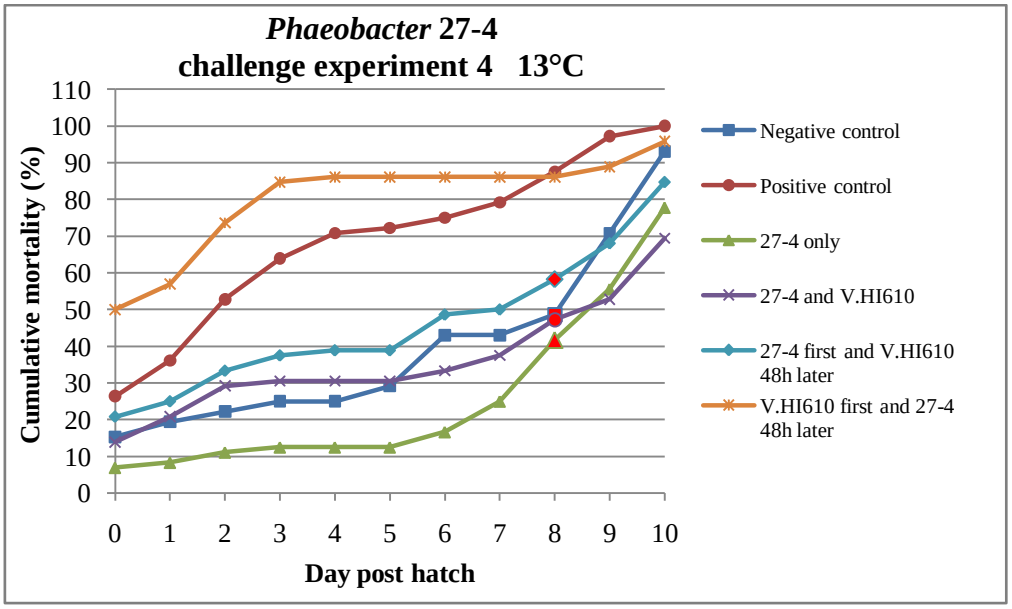

Figure 3.4.4 e): Percentage of cumulative mortality of non-feeding cod larvae challenged with *Phaeobacter* 27-4 and *V.anguillarum* HI610, alone, together at the same time and with a time laps of 48hours. All challenge doses are approximately high doses (10^6 CFU ml^{-1}). Negative control has no challenge dose added. Day 0: day of hatching. Red symbols (4): mortality rates 8dph significantly different from the positive control group, p-value (p<<0,001).

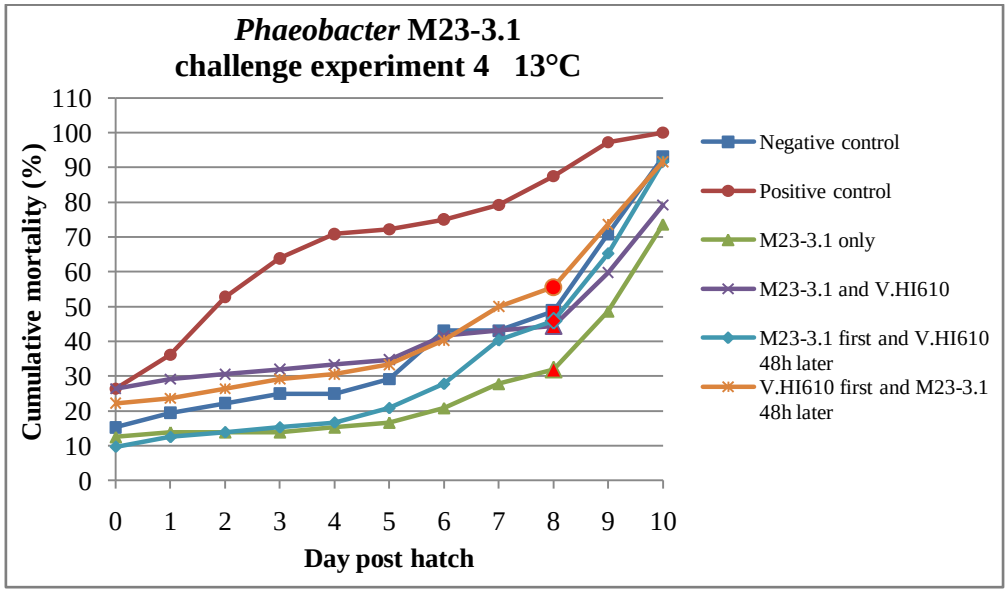

Figure 3.4.4 f): Percentage of cumulative mortality of non-feeding cod larvae challenged with *Phaeobacter* M23-3.1 and *V.anguillarum* HI610, alone, together at the same time and with a time laps of 48hours. All challenge doses are approximately high doses (10^6 CFU ml^{-1}). Negative control has no challenge dose added. Day 0: day of hatching. Red symbols (5): mortality rates 8dph significantly different from the positive control group, p-value (p<<0,001).

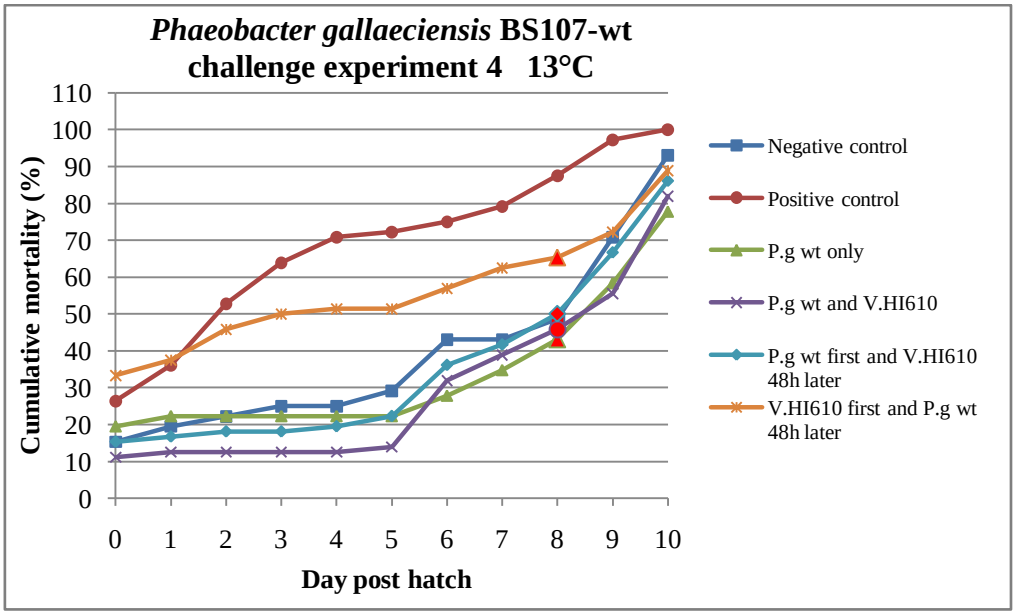

Figure 3.4.4 g): Percentage of cumulative mortality of non-feeding cod larvae challenged with *Phaeobacter gallaeciensis* BS107-wt and *V.anguillarum* HI610, alone, together at the same time and with a time laps of 48hours. All challenge doses are approximately high doses (10^6 CFU ml^{-1}). Negative control has no challenge dose added. Day 0: day of hatching. Red symbols (4): mortality rates 8dph significantly different from the positive control group, p-value (p<<0,001).

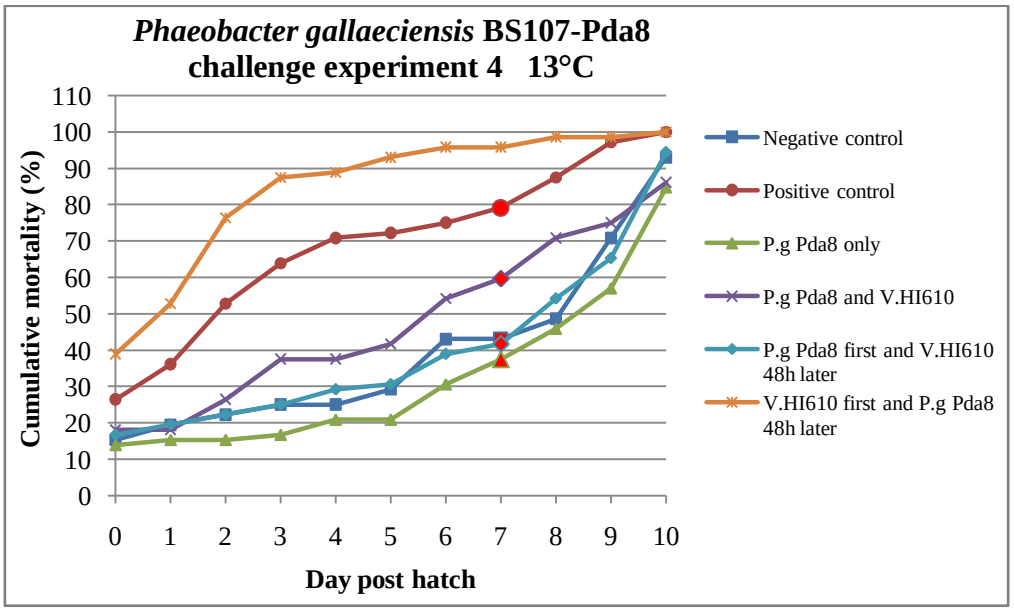

Figure 3.4.4 h): Percentage of cumulative mortality of non-feeding cod larvae challenged with *Phaeobacter gallaeciensis* BS107-Pgd8 and *V.anguillarum* HI610, alone, together at the same time and with a time laps of 48hours. All challenge doses are approximately high doses (10^6 CFU ml^{-1}). Negative control has no challenge dose added. Day 0: day of hatching. Red symbols (5): mortality rates 8dph significantly different from the positive control group, p-value (p<<0,001).

3.4.5. Challenge experiment number five

In challenge experiment number five at 7°C the cumulative mortality is shown in figure 3.4.5 a) – 3.4.5 d). The negative control group shows little mortality and is significant ($p<<0,001$) different from the positive control group. The groups with only probiotics and the groups with probiotic added 48 hours before *V.anguillarum* HI610 shows little mortality and are significantly ($p<<0,001$) different from the positive control group. In the groups were the probiotic is added at the same time as the pathogen there are differences in the cumulative mortality. *Phaeobacter* M23-3.1 and *V.anguillarum* HI610, and *Phaeobacter gallaeciensis* BS107-wt and *V.anguillarum* HI610 shows a lower mortality and are significantly ($p<<0,001$) different from the positive control group, in contrast to *Phaeobacter* 27-4 and *V.anguillarum* HI610, and *Phaeobacter gallaeciensis* BS107-Pda8 and *V.anguillarum* HI610 which shows no significant ($p >1$) difference from the positive control group. In all the groups were the *V.anguillarum* HI610 are added 48 hours before the probiotic the mortality was high and there were no significant ($p >1$) differences from the positive control group.

In challenge experiment number five at 13°C the cumulative mortality is shown in figure 3.4.5 e) – 3.4.5 h). The results in the 13°C parallel follows the 7°C parallel with only two deviations. The challenge group M23-3.1 and V.HI610 have high mortality and was not significant different from the positive control, the same result were for the challenge group P.g wt and V.HI610.

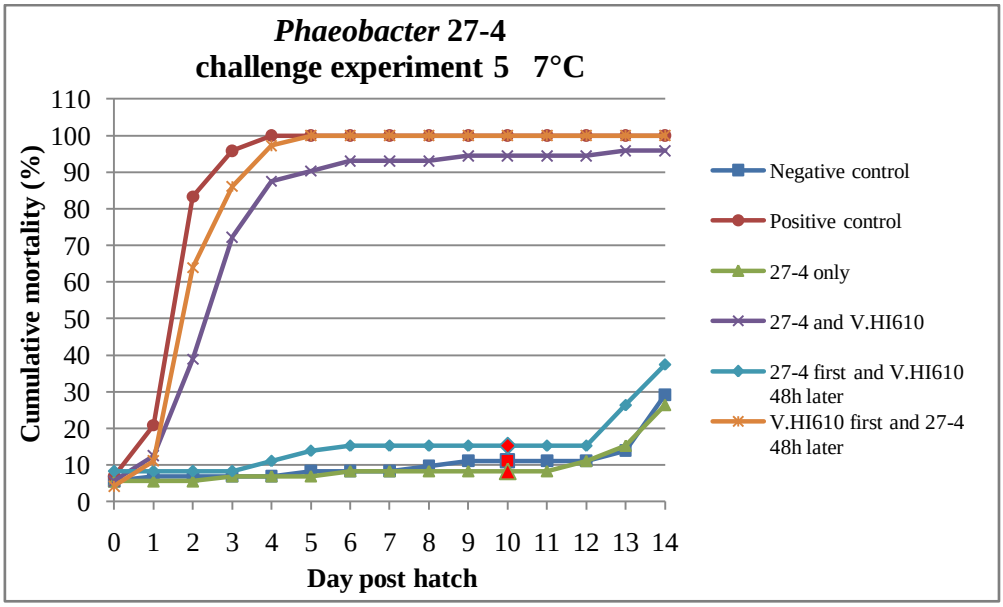

Figure 3.4.5 a): Percentage of cumulative mortality of non-feeding cod larvae challenged with *Phaeobacter* 27-4 and *V.anguillarum* HI610, alone, together at the same time and with a time laps of 48hours. All challenge doses are approximately high doses (10^6 CFU ml^{-1}). Negative control has no challenge dose added. Day 0: day of hatching. Red symbols (3): mortality rates 10dph significantly different from the positive control group, p-value (p<<0,001).

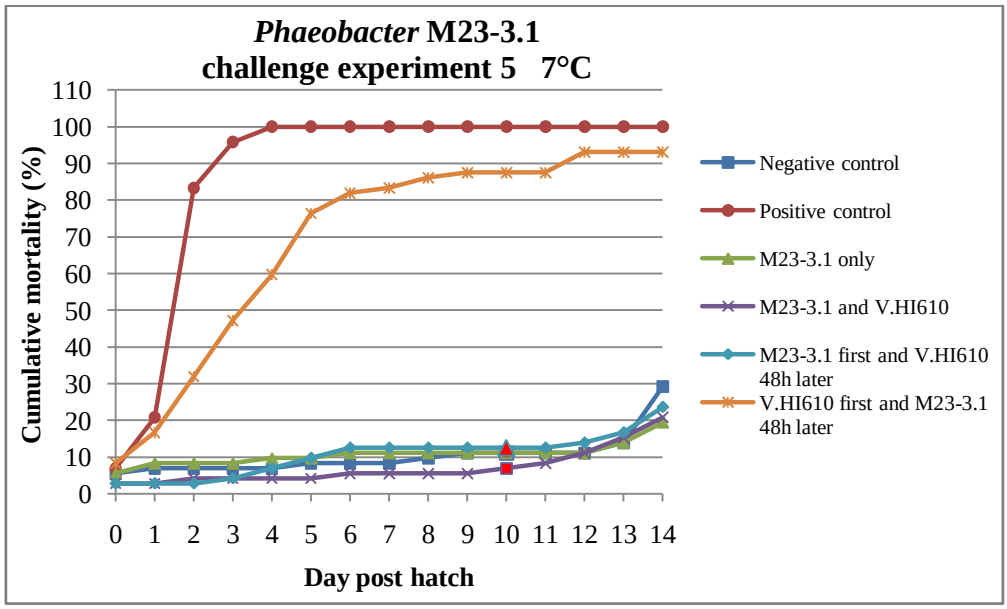

Figure 3.4.5 b): Percentage of cumulative mortality of non-feeding cod larvae challenged with *Phaeobacter* M23-3.1 and *V.anguillarum* HI610, alone, together at the same time and with a time laps of 48hours. All challenge doses are approximately high doses (10^6 CFU ml^{-1}). Negative control has no challenge dose added. Day 0: day of hatching. Red symbols (4): mortality rates 10dph significantly different from the positive control group, p-value (p<<0,001).

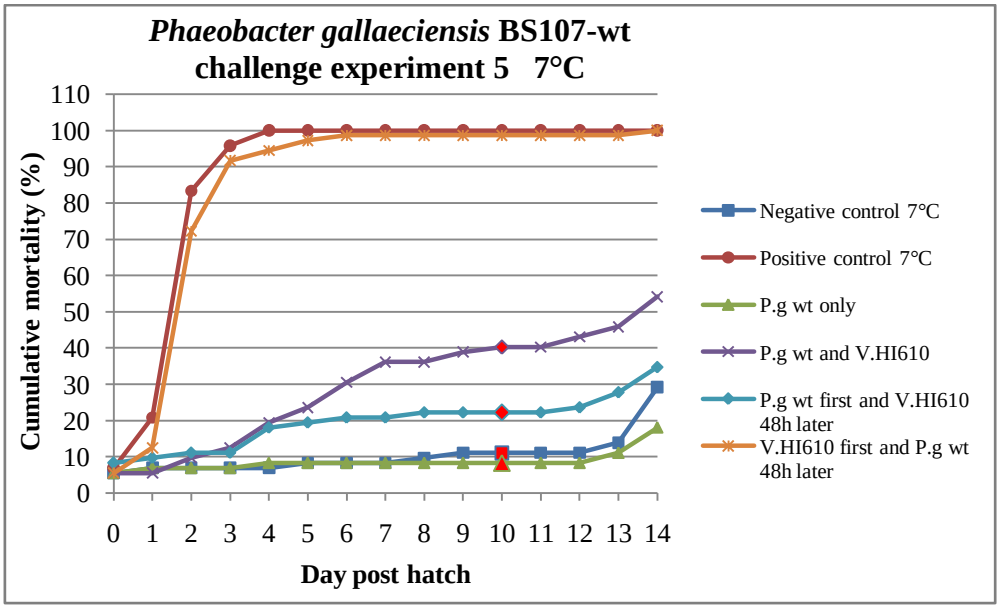

Figure 3.4.5 c): Percentage of cumulative mortality of non-feeding cod larvae challenged with *Phaeobacter gallaeciensis* BS107-wt and *V.anguillarum* HI610, alone, together at the same time and with a time laps of 48hours. All challenge doses are approximately high doses (10^6 CFU ml^{-1}). Negative control has no challenge dose added. Day 0: day of hatching. Red symbols (4): mortality rates 10dph significantly different from the positive control group, p-value (p<<0,001).

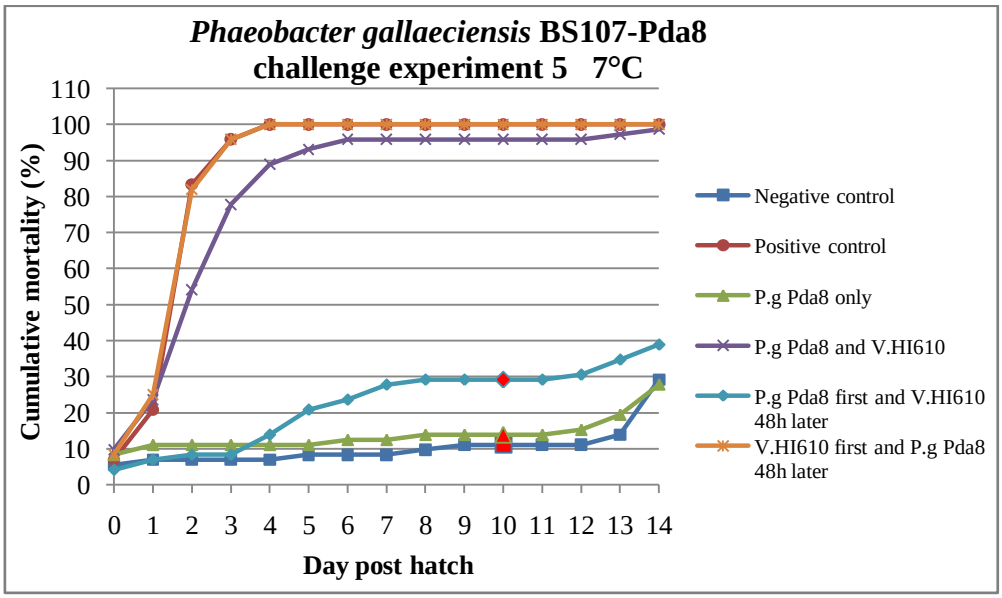

Figure 3.4.5 d): Percentage of cumulative mortality of non-feeding cod larvae challenged with *Phaeobacter gallaeciensis* BS107-Pgd8 and *V.anguillarum* HI610, alone, together at the same time and with a time laps of 48hours. All challenge doses are approximately high doses (10^6 CFU ml^{-1}). Negative control has no challenge dose added. Day 0: day of hatching. Red symbols (3): mortality rates 10dph significantly different from the positive control group, p-value (p<<0,001).

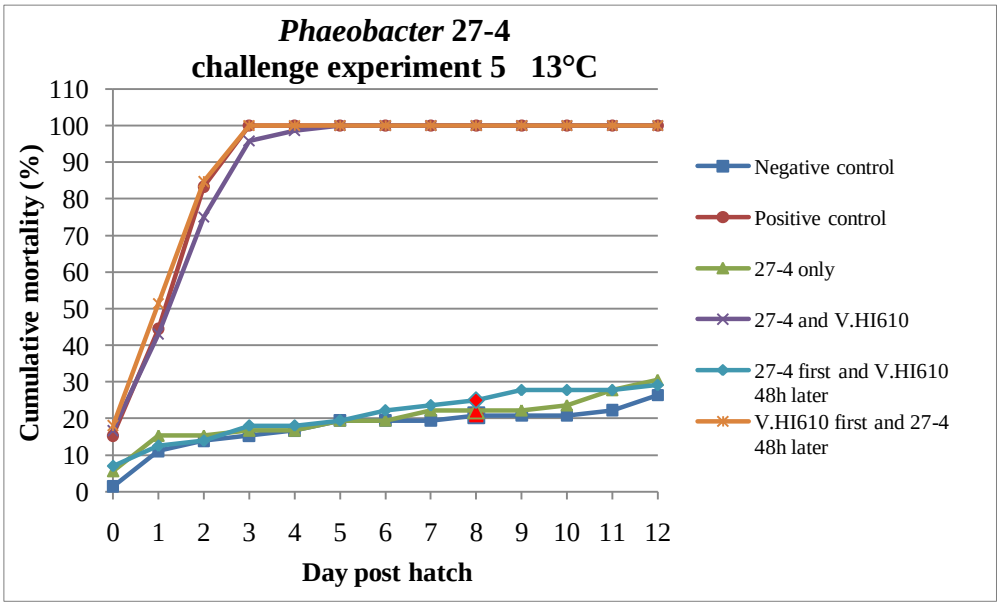

Figure 3.4.5 e): Percentage of cumulative mortality of non-feeding cod larvae challenged with *Phaeobacter* 27-4 and *V.anguillarum* HI610, alone, together at the same time and with a time laps of 48hours. All challenge doses are approximately high doses (10^6 CFU ml^{-1}). Negative control has no challenge dose added. Day 0: day of hatching. Red symbols (3): mortality rates 8dph significantly different from the positive control group, p-value (p<<0,001).

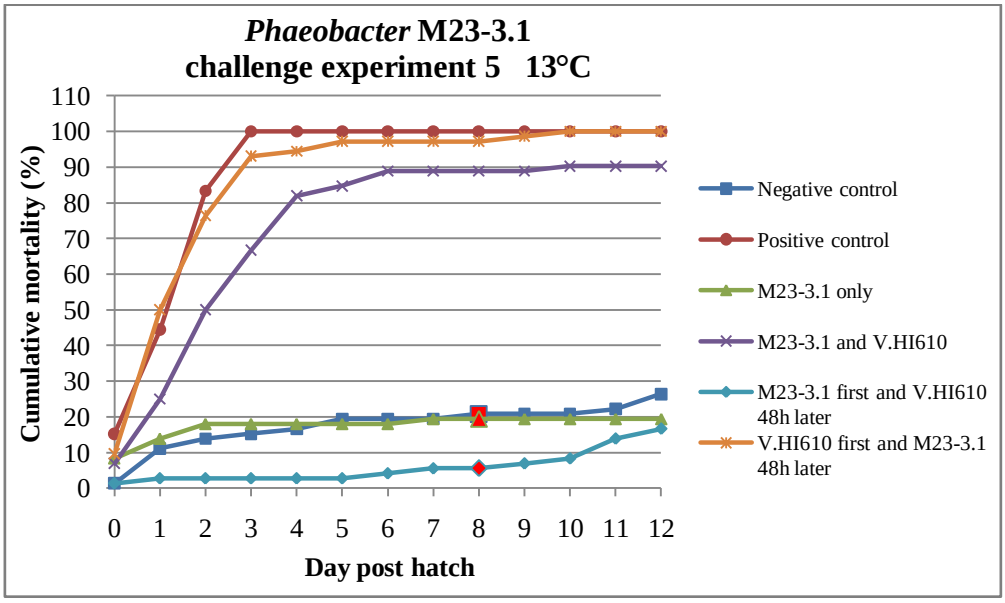

Figure 3.4.5 f): Percentage of cumulative mortality of non-feeding cod larvae challenged with *Phaeobacter* M23-3.1 and *V.anguillarum* HI610, alone, together at the same time and with a time laps of 48hours. All challenge doses are approximately high doses (10^6 CFU ml^{-1}). Negative control has no challenge dose added. Day 0: day of hatching. Red symbols (3): mortality rates 8dph significantly different from the positive control group, p-value (p<<0,001).

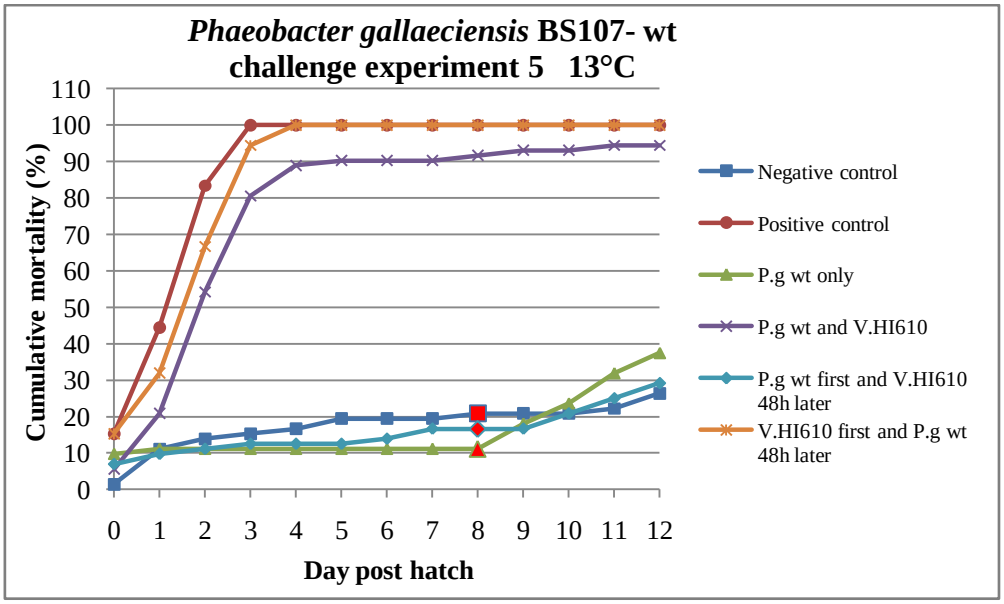

Figure 3.4.5 g): Percentage of cumulative mortality of non-feeding cod larvae challenged with *Phaeobacter gallaeciensis* BS107-wt and *V.anguillarum* HI610, alone, together at the same time and with a time laps of 48hours. All challenge doses are approximately high doses (10^6 CFU ml^{-1}). Negative control has no challenge dose added. Day 0: day of hatching. Red symbols (3): mortality rates 8dph significantly different from the positive control group, p-value (p<<0,001).

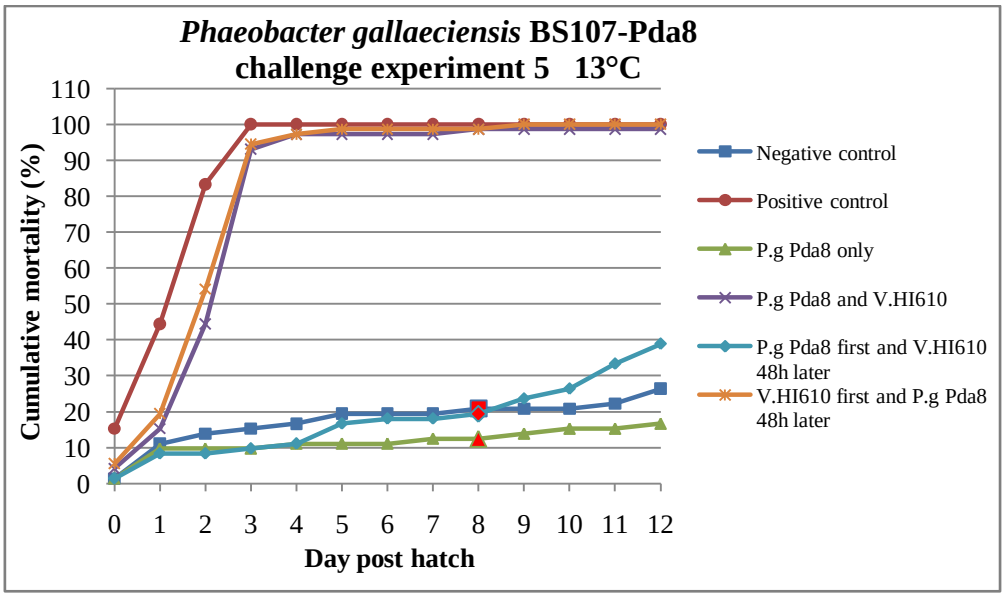

Figure 3.4.5 h): Percentage of cumulative mortality of non-feeding cod larvae challenged with *Phaeobacter gallaeciensis* BS107-Pgd8 and *V.anguillarum* HI610, alone, together at the same time and with a time laps of 48hours. All challenge doses are approximately high doses (10^6 CFU ml^{-1}). Negative control has no challenge dose added. Day 0: day of hatching. Red symbols (3): mortality rates 8dph significantly different from the positive control group, p-value (p<<0,001).

3.4.6. Challenge experiment number five, comparing 7°C versus 13°C

To compare possible differences between the 7°C experiments and the 13°C experiments the groups positive control, negative control, M23-3.1 only, M23-3.1 and V.HI610, M23-3.1first and V.HI610 48 later and V.HI610first and M23-3.1 48h later groups from both temperature regimes were put up against each other in an graph, figure 3.4.6 and in statistical analysis, p-value in table 3.4.6. There were not found any statistical differences between the different temperature groups, except from in two of the groups. The 7°C M23-3.1 and V.HI610 group is significant different ($p<<0,001$) from the 13°C M23-3.1 and V.HI610 group. The results indicates that the 7°C M23-3.1 and V.HI610 group has much lower cumulative mortality than the 13°C M23-3.1 and V.HI610 group, at 8 days post hatch. There are also significantly differences ($p<0,05$ / $p=0,035$) between the 7°C "V.HI610first and M23-3.1 48h later" group and the 13°C "V.HI610first and M23-3.1 48h later" group. The results show that the 7°C "V.HI610first and M23-3.1 48h later" group has significant lower mortality than the 13°C "V.HI610first and M23-3.1 48h later" group.

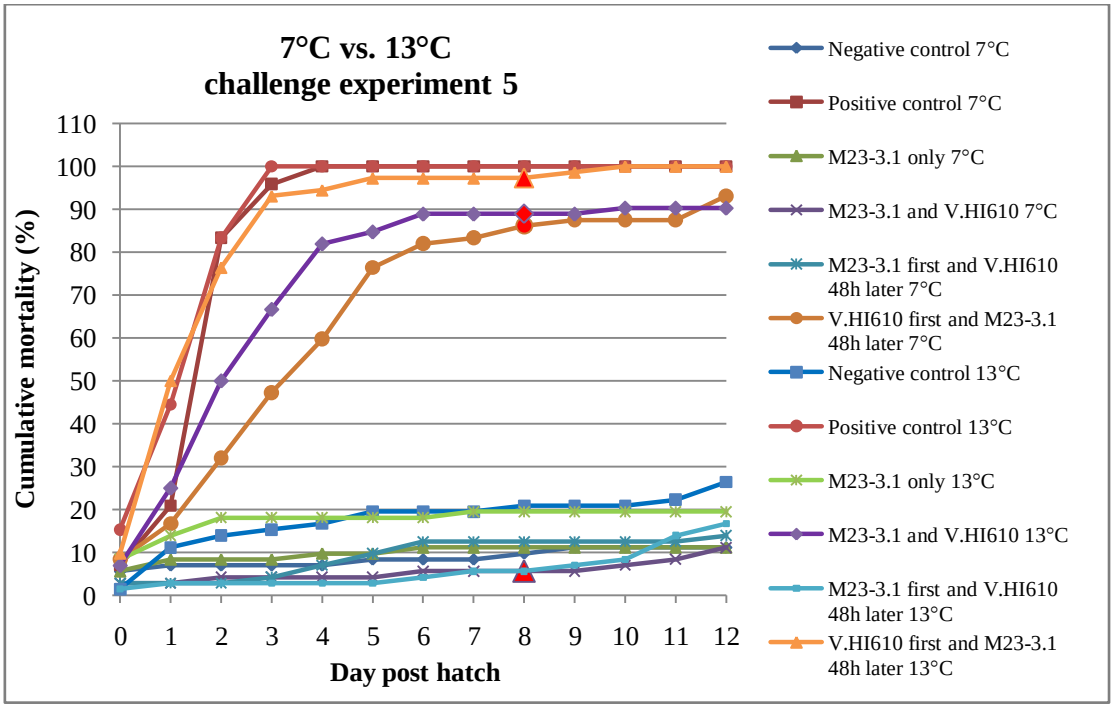

Figure 3.4.6: Percentage of cumulative mortality of non-feeding cod larvae against days post hatch in experiment number five. Comparison between cumulative mortality in the 7°C and 13°C positive control, negative control, M23-3.1 only, M23-3.1 and V.HI610, M23-3.1first and V.HI610 48h later and V.HI610first and M23-.3.1 48h later groups at 8 days post hatch. Day 0: day of hatching. Red symbols (4): significantly difference between the "M23-3.1 and V.HI610" groups ($p<<0,001$) and the "V.HI610first and M23-3.1 48 later" groups ($p<0,05$ / $p=0,035$).

Table 3.4.6: Table of the calculated p-value for the comparisons between temperatures and for the comparisons between hatchery A and hatchery B. C.m.% = Cumulative mortality in %. χ^2 = Yates-corrected Chi square values, p-value is significant if $p < \alpha < 0,05$. All significant p-values are showed in bold.

Strain	C.m. (%)	C.m. (%)	χ^2	**p-value**
temp vs. temp, 7°C vs. 13°C				
NC vs. NC	9,72	20,83	2,63	0,1049
PC vs. PC	100	100	Inf	-
M23-3.1 only vs. M23-3.1 only	11,11	19,44	1,34	0,2468
M23-3.1 and HI610 vs. M23-3.1 and HI610	5,56	88,89	96,99	**$2,2 \times 10^{-16}$**
M23-3.1first and HI610 48h after vs. M23-3.1first and HI610 48h after	12,50	5,56	1,35	0,2448
HI610first and M23-3.1 48h after vs. HI610first and M23-3.1 48h after	86,11	97,22	4,45	**0,0348**
hatchery A vs. hatchery B temp. 7°C				
NC vs. NC	20,84	11,11	1,86	0,1723
PC vs. PC	100	100	Inf	-
hatchery A vs. hatchery B temp. 13°C				
NC vs. NC	43,06	20,83	7,19	**0,0073**
PC vs. PC	100	100	Inf	-

3.4.7. Challenge experiment number five, comparing hatchery A versus hatchery B

In order to eliminate if there were differences between the egg quality from the two different hatcheries, A and B, there was done a comparison between the hatcheries negative control groups and between the positive control groups at the different temperatures 7°C and 13°C, at respectively 10days post hatch and 8days post hatch. In the 7°C comparison there were no significant differences between the positive control groups or the negative control groups. In the 13°C comparison there were no significant differences between the positive control groups, bur for the negative control groups there were significant differences in mortality at 8 days post hatch. The hatchery B negative control group had a slightly lower mortality than the hatchery A negative control group. Statistical calculations in table 3.4.6.

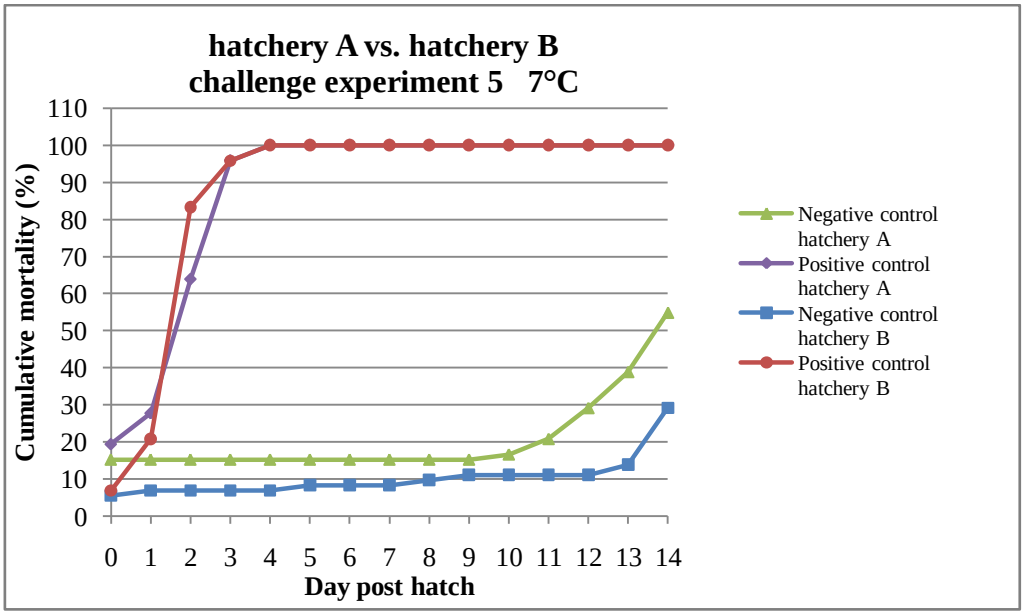

Figure 3.4.7 a): Percentage of cumulative mortality of non-feeding cod larvae in challenge experiment number five at 7°C. Negative and positive control groups from hatchery A versus negative and positive control groups from hatchery B. The positive control is high dose (10^6 CFU ml^{-1}). Negative control has no challenge dose added. Day 0: day of hatching. Mortality rates 10dph, there were no significantly differences between the hatcheries, p-value (p = 0,17) and (p > 1).

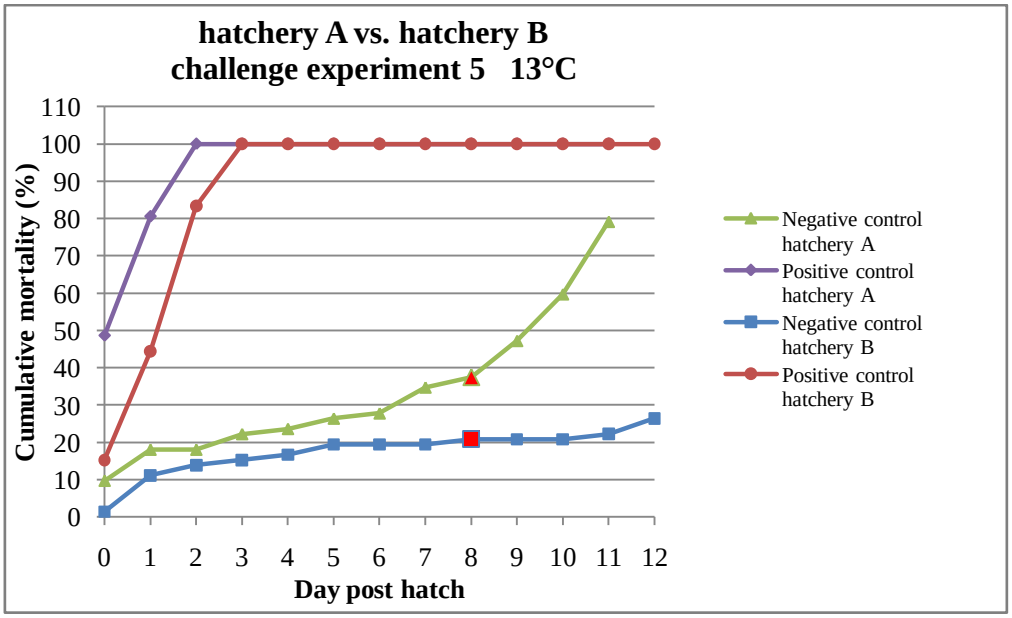

Figure 3.4.7 b): Percentage of cumulative mortality of non-feeding cod larvae in challenge experiment number five at 13°C. Negative and positive control groups from hatchery A versus negative and positive control groups from hatchery B. The positive control is high dose (10^6 CFU ml^{-1}). Negative control has no challenge dose added. Day 0: day of hatching. Red symbols (2): mortality rates 8dph, there were significantly difference between the negative controls from hatchery A and B, p-value (p<0,05) (p = 0,007). There was no significantly difference between the positive controls.

3.4.8 Other statistical comparisons

Table of the other statistical comparisons results are to be found in table 3.4.8 after this text.

Statistical comparisons were performed between the negative control group and the groups with only probiotics for the challenge experiments 3, 4 and 5 at 7°C and 13°C. The results shows that there are significant differences ($p<0,03$ and $p<<0,001$), lower mortality, in the groups which have probiotic present than in the negative control group, in experiment 3 and 4. However, there were no significant difference between these groups in experiment five and the 13°C experiments.

There were done statistical comparison between the groups were *V.anguillarum* HI610 were added 48hrs before the probiotic, and where the probiotics were added 48hrs before the *V.anguillarum* HI610 for experiments 3, 4 and 5 at both 7°C and 13°C. These results indicated that there were significant differences ($p<<0,001$) between those groups in experiment number five, both at 7°C and 13°C. In experiment number four at 7°C there were significant differences between 3 out of 4 groups, there is no significant differences between the "V.HI610 first and M23-3.1 48h later" group and the "M23-3.1 first and V.HI610 48h later" group. In experiment number four at 13°C there were significant differences ($p<<0,001$) between 2 out of 2 groups, there are no significant differences between the "V.HI610 first and M23-3.1 48h later" group and the "M23-3.1 first and V.HI610 48h later" group and between the "V.HI610 first and P.g wt 48h later" group and the "P.g wt first and V.HI610 48h later" group.

In challenging experiment number 3, 7°C (there were no 13°C/15°C experiment) there were significant difference ($p<<0,001$) between 3 out of 4 groups. There is no significant difference between the "V.HI610 first and M23-3.1 48h later" group and the "M23-3.1 first and V.HI610 48h later" group.

Table 3.4.8: Table of statistical differences in cumulative mortality between the challenge groups in experiment 3, 4 and 5 at 10 days post hatch for 7°C and 8 days post hatch for 13°C. C.m.(%) is cumulative mortality in percent. χ^2 = Yates-corrected Chi square values, p-value is significant if $\alpha < 0,05$. All significant p-values are showed in bold.

Strain	challenge experiment no. 3 10/8 dph C.m. (%)	C.m. (%)	χ^2	p-value	challenge experiment no. 4 10/8 dph C.m. (%)	C.m. (%)	χ^2	p-value	challenge experiment no. 5 10/8 dph C.m. (%)	C.m. (%)	χ^2	p-value
Temperature 7°C												
NC vs. only 27-4	30,56	12,50	5,92	**0,01**	44,44	27,78	3,64	0,06	11,11	8,33	0,08	0,78
NC vs. only M23-3.1	30,56	19,44	1,81	0,18	44,44	11,11	18,31	**$1{,}88 \times 10^{-5}$**	11,11	11,11	0,07	0,79
NC vs. only P.g wt	30,56	11,11	7,12	**0,01**	44,44	9,72	20,25	**$6{,}78 \times 10^{-6}$**	11,11	8,33	0,08	0,78
NC vs. only P.g Pda8	30,56	13,89	4,86	**0,03**	44,44	16,67	11,81	**0,0006**	11,11	13,89	0,06	0,80
HI610first and 27-4 48h after vs. 27-4first and HI610 48h after	97,22	50	38,93	**$4{,}389 \times 10^{-10}$**	97,22	23,61	78,52	**$2{,}2 \times 10^{-16}$**	100	15,28	102,39	**$2{,}2 \times 10^{-16}$**
HI610first and M23-3.1 48h after vs. M23-3.1first and HI610 48h after	16,67	13,89	0,05	0,8168	19,44	12,50	0,83	0,36	87,50	12,50	78,03	**$2{,}2 \times 10^{-16}$**
HI610first and P.g wt 48h after vs. P.g wt first and HI610 48h after	73,61	16,67	44,87	**$2{,}107 \times 10^{-11}$**	69,44	13,89	43,46	**$4{,}33 \times 10^{-11}$**	98,61	22,22	84,67	**$2{,}2 \times 10^{-16}$**
HI610first and P.g Pda8 48h after vs. P.g Pda8 first and HI610 48h after	100	40,28	58,49	**$2{,}045 \times 10^{-14}$**	100	15,28	102,39	**$2{,}2 \times 10^{-16}$**	100	29,17	75,90	**$2{,}2 \times 10^{-16}$**
Temperature 13°C												
NC vs. only 27-4					48,61	41,67	0,45	0,50	20,83	22,22	0,00	1
NC vs. only M23-3.1					48,61	31,94	3,49	0,06	20,83	19,44	0,00	1
NC vs. only P.g wt					48,61	43,06	0,25	0,62	20,83	11,11	1,86	0,17
NC vs. only P.g Pda8					48,61	45,83	0,03	0,87	20,83	12,50	1,25	0,26
HI610first and 27-4 48h after vs. 27-4first and HI610 48h after					86,11	58,33	12,50	**0,0004**	100	25	83,23	**$2{,}2 \times 10^{-16}$**
HI610first and M23-3.1 48h after vs. M23-3.1first and HI610 48h after					65,28	50	1,00	0,32	97,22	5,56	117,45	**$2{,}2 \times 10^{-16}$**
HI610first and P.g wt 48h after vs. P.g wt first and HI610 48h after					50	45,83	2,84	0,09	100	16,67	99,46	**$2{,}2 \times 10^{-16}$**
HI610first and P.g Pda8 48h after vs. P.g Pda8 first and HI610 48h after					98,61	54,17	37,00	**$1{,}181 \times 10^{-9}$**	98,61	19,44	90,05	**$2{,}2 \times 10^{-16}$**

3.5 Gram staining pictures

In order to obtain pictures of the rosettes formed by the probiotic bacteria, Gram staining and fluorescent microscopy (ZEISS, Axioskop 2plus) was done.

Gram staining preparation were made out of 6 of the 8 probiotics that are included in this thesis, *Phaeobacter* 27-4, mutant JBB1001, *Pseudoalteromonas citrea* AQ10, *Phaeobacter* M23-3.1, *Phaeobacter gallaeciensis* BS107-wt and *Phaeobacter gallaeciensis* BS107-Pda8.

In figure 3.5 each probiotic that were Gram stained are shown. In all the pictures, except picture c), distinct rosette shaped clusters of bacteria are shown. In picture c) there are single rod shaped bacteria, this is the *Pseudoalteromonas citrea*, which is not known to make rosettes or cluster together.

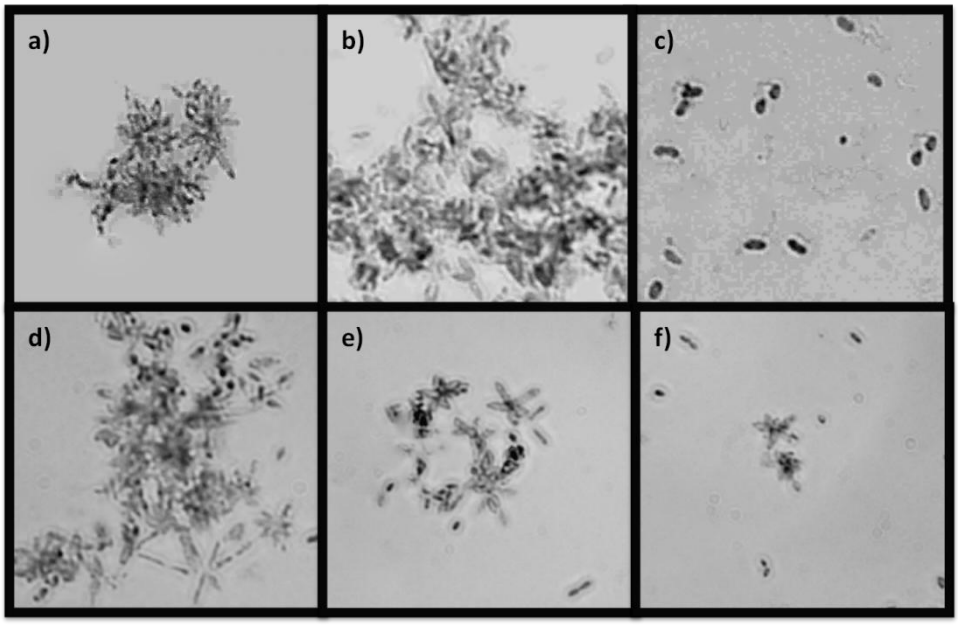

Figure 3.5: Pictures of gram staining preparations of 6 probiotics taken with a fluorescent microscopy (ZEISS, Axioskop 2plus) with bright-field illumination and a connected camera. a) *Phaeobacter* 27-4, b) mutant JBB1001, c) AQ10 *Pseudoalteromonas citrea*, d) *Phaeobacter* M23-3.1, e) *Phaeobacter gallaeciensis* BS107-wt, f) *Phaeobacter gallaeciensis* BS107-Pda8.

4 Discussion

The cultivation of marine larvae is known to struggle with high mortality due to high density of larvae and have high density of opportunistic pathogens. Today, globally, antibiotics are still used to prevent and treat bacterial infections in marine fish larvae, because the larvae have an immature immune system, and cannot be vaccinated. The antibiotic consumption is not favourable for the environment and there is a high risk for developing resistant bacteria. Due to this there is a need for alternative treatments against bacterial pathogens in the early life stages of marine larvae. The use of probiotics as an alternative to prevent and reduce bacterial diseases has become a current interest and there is intense research around the evaluation and use of probiotic bacteria.

The so-called *Roseobacter* clade commonly found in the marine environment include the genera *Phaeobacter* and *Ruegeria* which both include species that have been suggested as probiotic organisms.

The aim of the present study was by *in vivo* challenge experiments to enhance our knowledge of different probiotic used as a health benefit for the cod larvae, and as an antagonist against *Vibrio anguillarum*. The probiotic tested against *Vibrio anguillarum* were mainly *Phaeobacters*, but also *Ruegeria* and one *Pseudoalteromonas citrea*. Results from the different challenge experiments showed that the probiotics tested had a positive effect of the survival of the cod larva in general without *V.anguillarum* added, and with the pathogen present.

As a starting point it is highly important that the eggs used in a challenge experiment are of good quality and have good hatching percent and survival percent, because low quality could affect the results of the experiments.
The egg batches for the five challenge experiments were delivered from two different hatcheries, hatchery A and hatchery B. For challenge experiment 1, 3 and 4 the eggs were delivered from hatchery A and for challenge experiment 2 and 5 the eggs were delivered from hatchery B. The eggs were transported by car, plane and/or boat over several hours. Cod eggs are known to have varying percentage of hatching (Kjørsvik, 1994, Moksness et al., 2004) this in addition to the stress from the transport time, transport vehicle, possible temperature

changes under transport, the possibility for physical damage from handling the eggs after arrival and the temperature leap from the transport water and the water temperature in the wells could have an impact on the hatching percentage and give a delay in the hatching process. Stress and higher water temperatures results in lower oxygen and higher nitrogen, and the embryo development are highly temperature dependent and big differences in temperatures could result in higher mortality and deformed larvae (Kjørsvik, 1994, Moksness et al., 2004). To avoid that the intra-batch varieties could show in specific groups with an effect in the hatching percent and cumulative mortality, the eggs were randomized.

The challenge doses used during this experiment were approximately 10^6 colony forming units (CFU) ml^{-1}, which caused high mortality in the positive control groups with *Vibrio anguillarum* HI610. For the probiotics the exact challenge doses were difficult to estimate as exactly as the one for the *Vibrio*, but all the probiotic doses are estimated to be approximately high dose 10^6 CFU ml^{-1} or higher.

The difficulties to estimate the exact challenge dose concentration for the probiotics might be because of the lack of a good and certain procedure to estimate probiotic in a medium and on agar plates. Much of the difficulties might be due to the characteristic that many of the probiotics cluster together in rosette formations (Porsby et al., 2008). The clustering and rosettes formations were the reason that the counting chamber technique could not be used properly. We also experienced difficulties when trying to separate the bacteria. There were difficulties separating the bacteria, with EDTA, Ethylenediaminetetraacetic acid. Due to the rosette formations, the colony forming units on Marine agar-plates probably underestimated the CFU in some cases. However, the CFU protocol together with OD in lye, NaOH, was used as the procedure to perform the probiotic challenge dose. The procedure was done by exactly the same protocol in each experiment, to reduce the uncertainty for different challenge dose concentrations. However, even with the exact same procedure every time we experienced some differences in challenge dose concentration as measured by OD. The differences might be due to the rosettes and that they are hard to separate, this makes the OD measuring difficult.

There are other uncertainties with the challenge dose concentration, as contamination and viability of the bacteria after being washed and handled, if the challenge dose is the same in each well and the leap in concentration when challenge doses for the same experiment are made two times with an interval of 48 hours. These uncertainties make it even more important

to follow the exact same procedure every time and use proved sterile lab techniques, to limit the source of error. Another thing that could explain the challenge dose concentrations difficulties are that the growth curves are difficult to establish with good optimal OD, because of the cultivations properties, no shaking and a new bulb with culture at each new measure.
When one of the challenge bacteria, the pathogen or the probiotic are added 48hours before the other, the first bacteria added has an advantage with the extra time to settle in the environment. The first bacteria added would have a higher possibility to "succeed". If the pathogen was added first it would have a higher possibility to infect and kill the cod larvae, while when the probiotic was added first the probiotic would have a better possibility to outcompete the pathogen added 48 hours after.

The multi-well dish system is an easy and uncomplicated way to do *in vivo* challenge experiments, but some complications could occur. When the eggs are incubated in the wells some water will follow from the transport container, and as the method used to transfer the eggs is not sterile, and the eggs possess an epibiotic microflora (reviewed by (Hansen and Olafsen, 1999), this microflora could inoculate the water in the wells and "compete" with the bacteria added by challenge. The epibiotic microflora following the eggs might have an impact on the mortality results of the challenge experiments. Two of the batches delivered from hatchery A, (the once for challenge experiment number four and five) were treated with Buffodine (Evans Vanodine International Plc) before they sent the eggs from the hatchery, and this might have had some effect minimizing the bacterial flora already established in the rearing water. It is likely that development of gnotobiotic protocols, as done for sea bass larvae cited by Dierckens et al., 2009 would help to overcome this problem.
The early day post hatch mortality registered in challenge experiment number one could be explained by the following microflora, the handling of the eggs, transportation and the distribution to wells (first time distributing eggs to wells, therefore missed having eggs in some wells).
Any challenge method is to some extent artificial. However the bath challenge method mimics the natural challenge situation, where the larvae hatches in an environment to which bacteria has already been added (experimental situation) - or are present (natural situation).

In our experiments the positive control groups larvae were challenged with *Vibrio anguillarum* strain HI610 added through water, a method that previously has shown to cause mortality of Atlantic Cod, (*Gadus morhua*) (Samuelsen and Bergh, 2004, Seljestokken et al.,

2006, Vik-Mo et al., 2005). High and rapid mortality was also the results for all of the positive control groups for all the challenge experiments performed here. As early as 3 days post hatch nearly 100% of the larvae were dead in the positive control groups. This was a significantly higher mortality than all the other challenging groups except the groups where the *Vibrio* strain were added 48hours before the probiotic strains. These challenge groups indicated the same high and early cumulative mortality as the positive control groups when the *V.anguillarum* were challenged together with the *Phaeobacter* 27-4 and the *Phaeobacter gallaeciensis* BS107-Pda8 (mutant) in the respective experiments. In the group where the *Vibrio* strain were added 48 hours before the probiotic strain *Phaeobacter* M23-3.1, the mortality were significantly lower than the other similar groups, where the *Vibrio* strain were added 48 hours before the probiotics *Phaeobacter* 27-4, *Phaeobacter gallaeciensis* BS107-wt and *Phaeobacter gallaeciensis* BS107-Pda8 (mutant) . In the group where the *Vibrio* strain were added 48 hours before the probiotic strain *Phaeobacter gallaeciensis* BS107-wt the mortality were also significantly lower (except from in challenge experiment number five) than the positive control, but not as low as with the probiotic strain *Phaeobacter* M23-3.1. This indicates that some probiotic strains may have a more potent probiotic effect than others.

Challenge experiment number one where in some extent a test experiment, a first trial. Therefore I reduced the presented results in one figure, figure 3.4.1 under Results. From the graph one could see high cumulative mortality early (in time aspect) after 0dph for the high dose positive control, as wanted. The low dose positive control shows a much lower mortality, indicating that the 10^4 CFU ml^{-1} might be too low to cause the wanted significantly mortality. Because of these results the low dose positive control were excluded from the next experiments. All the probiotic challenge doses were high doses, approximately 10^6 CFU ml^{-1}. The challenge groups with probiotics alone *Ruegeria* F1926 and *Ruegeria* M43-2.3 showed a generally low cumulative mortality, in line with the negative control. The probiotics F1926 and M43-2.3 were tested up against the low dose *V.anguillarum* HI610 at the same and with the probiotic 48hrs before the low dose *V.anguillarum* HI610. These groups all showed a low cumulative mortality. This became no surprise when the low dose showed such low mortality alone. The probiotics *Ruegeria* F1926 and *Ruegeria* M43-2.3 were only tested in this first challenge experiment due to that other probiotics were of more interest, and I had to choose which once I wanted to work with.

It is has previously been showed that larvae starved longer than 9 days post hatch cannot survive (Kjørsvik et al., 1991) and it is known from earlier studies using a multi-dish based protocol (Sandlund et al., 2010) that larvae which are not fed during the experiment will have a natural increase in mortality after 10 days post hatch, due to starvation. Since the larvae were not fed during these challenge experiments it is reasonable to conclude that the increase in mortality after 10 days post hatch is due to starvation.

In challenge experiment number two there were performed a 7°C challenge experiment and a parallel at 15°C, figures 3.4.2 a) and 3.4.2 b). In the 7°C challenge experiment the positive control and the challenge group with V.HI610 first and *Phaeobacter* 27-4 48hours after showed a high cumulative mortality, actually the group with V.HI610 first and *Phaeobacter* 27-4 48hours after shows a slightly higher mortality than the positive control 4dph and throughout the experiment, 8dph. There were some problems with the mortality registration for the 7°C experiment, and therefore the experiment was ended at 8dph. Because of the early conclusion, I did not do any statistical calculation for this experiment. However, the graph for the 7°C experiment clearly shows that all the other groups: negative control, 27-4 only, 27-4 and V.HI610, 27-4 first and V.HI610 48hrs after, M23-3.1 only, M23-3.1 and V.HI610, M23-3.1 first and V.HI610 and V.HI610 first and M23-3.1 48hrs after have much lower cumulative mortality than the positive control and the V.HI610 first and 27-4 48hrs after groups.
In challenge experiment number two the first parallel on 15°C was tested. In figure 3.4.2 b) one could see that all the challenge groups have an exponential increasing cumulative mortality. It seems like all the groups would end up in a 100% cumulative mortality some time after 10dph. The positive control and the groups with pathogen and *Phaeobacter* 27-4 are the groups reaching the 100% mortality first. This exponential increase in cumulative mortality might be due to the temperature leap and/or the extra handling of the eggs when increasing the temperature. In addition the increase of temperature on the eggs were done in the original transport water with aeration over 48hours, this increase in temperature over a short time leap could have boosted any bacteria flora already present and effected the embryo development and reduced the larvae capability of survival. In the later experiments 50% of the transport water was changed with 50% autoclaved sterile filtered and aerated diluted seawater (32‰) to see if this could reduce mortality after the temperature increase.

In challenge experiment number three at 7°C four probiotics were tested, *Phaeobacter* 27-4, *Phaeobacter* M23-3.1, *Phaeobacter gallaeciensis* BS107-wt, *Phaeobacter gallaeciensis* BS107-Pda8, figure 3.4.3 a), b) c) and d). The positive control showed a significantly higher mortality than all the other challenge groups tested, except from the "V.HI610 first and 27-4 48hrs after" group and the "V.HI610 first and P.g Pda8 48hrs after" group. The challenge groups with *Phaeobacter* M23-3.1 showed the most significant difference with the positive control followed by the challenge groups with *Phaeobacter gallaeciensis* BS107-wt. This result again, proves that the Phaeobacter M23-3.1 is a more potent probiotic.
The 15°C parallel in challenge experiment number five failed. The temperature was increased a whole eight degrees in only 24 hours. This was probably a much too fast increase and an increase that might have influenced the embryo development that caused a low hatching percent and a high number of deformed larvae in the few hatched eggs. The deformed larvae could again have an effect on the infection grade and the high cumulative mortality.

In challenge experiment number four at 7°C, figures 3.4.4 a), b), c) and d) there is "high" mortality in the negative control, over 40% cumulative mortality at 2dph while the challenge groups 27-4 only, 27-4 first and V.HI610 48hrs after, M23-3.1 only, M23-3.1 and V.HI610, M23-3.1 first and V.HI610 48hrs after, V.HI610 first and M23-3.1 48hrs after, P.g wt only, P.g wt and V.HI610, P.g wt first and V.HI610 48hrs after, P.g Pda8 only and P.g Pda8 first and V.HI610 48hrs after shows a significantly lower mortality. Earlier studies has also showed high mortality in the negative control groups (Hjelm et al., 2004). However, this result clearly shows that the probiotics have a positive effect on the survival when these groups show significantly lower mortality than the negative control that are supposed to be "bacteria free" and the group with the highest survival numbers.
In the 13°C parallel in challenge experiment number four the temperature increase did not seem to highly successful for the cumulative mortalities. The positive control group showed significantly higher mortality than all the other challenge groups at 8dph, except one, the V.HI610 first and 27-4 48hrs after, which cumulative mortality percent are almost the same as the positive control at 8dph. Despite the significantly higher mortality in the positive control the temperature increase were no good, since all the challenge groups' cumulative mortality curves have an exponential growth and seem to reach 100% mortality before, near or after 10dph. One conclusion is that the procedure for increasing the temperature on eggs has to be advanced to give the eggs a smoother transition between temperatures.

In challenge experiment number five, figures 3.4.5 a) – 3.4.5 h) we got some problems because of the too early hatching. Some eggs were already hatched when we randomised the eggs into wells the day of arrival. This resulted in that some eggs were hatched when I challenged the second time at 7°C, and a lot of eggs were hatched when I challenged the first time at 13°C, and over 50% were hatched when I challenged the second time at 13°C. This hatching before I finished the challenging might have an effect on the cumulative mortality, higher, lower or as the other experiments.
In the 7°C parallel it seems that the pathogen *V.anguillarum* HI610 had got a small advantage by the early hatching, when the *Vibrio* were added 48hrs before the probiotics. And the groups "27-4 and V.HI610" and "P.g Pda8 and V.HI610" were not significant different from the positive control.
In the 13°C parallel the *V.anguillarum* HI610 again seems to have a small advantage by the early hatching, when the *Vibrio* were added 48hrs before the probiotics and at the same time as the probiotics (see table 7.8 b) in Appendix 7.8 for significantly p-values). This could indicate that the infection rate for *Vibrio* is higher when the cod larva hatches earlier, or, alternatively, that the higher mortality rather is because of the handling/challenging (stress) of larvae instead of eggs.

There were done comparisons between the two temperature regimes in challenge experiment number five, showed in figure 3.4.6 under Results, with the groups' positive control, negative control, only M23-3.1, M23-3.1 and V.HI610, M23-3.1first and V.HI610 48hrs after and with V.HI610 first and M23-3.1 48hrs after. The results indicated that there were no significantly differences in mortality between the parallel temperature groups 7°C and 13°C, except from two groups. M23-3.1 and V.HI610 had a significantly lower mortality at 7°C than at 13°C. The group "V.HI610 first and M23-3.1 48hrs after", also indicated a significantly lower mortality at 7°C than at 13°C. This difference in mortality could be because of a natural lower hatching percentage or/and a natural higher mortality of larvae with increasing temperature, or that the higher temperature is nearer the *Vibrio* optimum temperature and the *Vibrio* got more virulent (Larsen et al., 2004). Despite some higher general mortality at 13°C the results from groups with probiotic added, still indicates good effect.

There were done visual observations of the eggs from hatchery A and hatchery B. The registrations were that the eggs from hatchery A were smaller, with lesser pigmentation and

they seemed to have lower hatching percent and shorter lifespan than the eggs from hatchery B. The hypotheses about the hatching percent and shorter lifespan was rejected by statistical comparisons of the two hatcheries in challenge experiment number five in both 7°C and 13°C. In challenge experiment number five we included two batches with cod eggs, one from hatchery A and one from hatchery B. The batch from hatchery B was used to perform the entire challenge experiment number five, i.e. all challenged groups were using eggs from this hatchery. The eggs from hatchery A were used to perform parallels of negative and positive control groups at both 7°C and 13°C against the negative and positive control groups in challenge experiment number five, thereby comparing the two egg batches. The graph, shown in figure 3.4.7 a) under Results, and the statistical comparisons, in table 3.4.6 under Results, indicated no differences between the positive or negative control groups at 7°C. At 13°C, figure 3.4.7 b) and table 3.4.6 under Results, there were no differences between the positive control groups, but a small but significant difference was found between the two negative control groups. There was a slightly higher mortality in the negative control group from hatchery A. This difference might have been due to the temperature leap and not to the quality of the eggs. The eggs from hatchery A had a lower transport temperature and cultivation temperature than the eggs from hatchery B, which gave a higher temperature leap for the eggs from hatchery A. Other conditions that could affect the results in the experiments are the transportation time, the transportation vehicle, the fact that the eggs derive from two different hatcheries and broodstocks populations with distinctly different genetic origin that gives them different morphology.

The time for registering mortality ought to be done at the same time every day, especially for the 13°C (15°C) because of the biological processes that are known to go faster at higher temperatures (Moksness et al., 2004) and to get a more precisely curve for cumulative mortality. It could sometimes be difficult to see the difference between dead and alive, and registration two times on 24 hours might have been a good idea to get a more precisely mortality curve. However, the extra handling could be a stress factor and cause sooner and higher mortality.

All the bacteria, the pathogen and the probiotic have different optimums that could affect the mortality rates, the pathogen, *Vibrio anguillarum* have high optimum temperatures, higher than 15°C (Larsen and Pedersen, 1999, Larsen et al., 2004) and the antimicrobial activity of TDA in probiotics is degraded at higher temperatures (Porsby, 2010).

In the performed challenge experiments a *Phaeobacter gallaeciensis* BS107-Pda8 mutant of the *Phaeobacter gallaeciensis* BS107-wt was tested. This *Phaeobacter gallaeciensis* BS107-Pda8 lack the gene for TDA, and theoretically the following antimicrobial activity and pigmentation ability (like the JBB1001 mutant in (Geng et al., 2008)). The lack of pigmentation was seen under cultivation, while the lack of antimicrobial activity should show under results in the graphs over cumulative mortality in figure 3.4.3 d), 3.4.4 d) and 3.4.5 d). These results shows that the challenge group P.g Pda8 only and the challenge group where the P.g Pda8 was added 48hrs before the V.HI610 was significantly lower in mortality than the positive control. While the challenge group where the P.g Pda8 is added together with V.HI610 at the same time and the challenge group where the V.HI610 is added 48hrs before the P.g Pda8 has no significant difference in mortality from the positive control.

5 Conclusion and suggestions for further work

In conclusion this study has demonstrated that the use of different strains of probiotics against the pathogen *Vibrio anguillarum* HI610 in an experimentally multi-dish bath challenge with cod yolk sac larvae, *Gadus morhua*, has an inhibitory effect against the pathogen measured in terms of impact of cod larvae survival following challenge. Furthermore, no negative or harmful effects of the probiotic additions could be found. This confirms and establishes earlier studies of the possible probiotic effect these strains *Phaeobacter* 27-4, *Phaeobacter* M23-3.1 and *Phaeobacter gallaeciensis* BS107-wt were believed to have. The study also demonstrated that some probiotic might have stronger probiotic effect than others. In this study, the probiotic *Phaeobacter* M23-3.1 that showed a higher probiotic effect with the lowest cumulative mortality days post hatch.

It seems that if this experiment had been allowed to continue longer there would be a continuance in high survival in the challenge groups were there were added probiotics alone, before and at the same time as the pathogen *V.anguillarum* HI610. This would have demanded feeding of the larvae and a subsequent change in bacteria flora following onset of feeding. The change in bacterial flora might have been a challenge for the probiotic bacteria, and the effect of this remains to be investigated.

Further investigation of the probiotic effect of these strains against pathogens has to include other pathogens, maybe pathogens that are considered as a bigger problem at the early larvae stages like the bacteria *Flexibacter, Flavobacterium* and *Cytophaga*, (Moksness et al., 2004), bath challenge of fed larva and maybe inoculation via live feed and challenge experiment in "normal" sized rearing facilities with water in circulation.

Literature and studies cited in (Porsby, 2010) have indicated that the some of the probiotics have lower probiotic effect when there are too much movement and circulation in the cultivation medium. Because of this it is important to investigate if there is any reduction in the probiotic effect in some like "normal" sized larvae cultivations. There must be done studies and work to adapt a technology that is suitable for the probiotic strains in question.

It could be interesting to study *in vitro* challenging experiments with the same pathogen and probiotics to see if the probiotics would give any internal effects and if there are temperature differences in mortality in such an experiment. By use of confocal microscopy and

GFP-transformed bacteria there might be a possibility to study if there is any uptake of pathogen and probiotic *in vitro* in the larvae.

However, the most interesting prospect in an economical and biological context is that the probiotics seem to be capable to outcompete pathogens and be utilised in the role as an antagonistic or antibiotics in regular larval rearing facilities.

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7 Appendix

7.1 Cultivation of the probiotics

Growth medium, Marine Broth

37,4gram (Difco™ 2216, Becton, Dickinson and Company) of the powder in 1liter with dH_2O (distilled water), mix well and heat or boil for 1min while stir.

Spread the broth in several 250ml Erlenmeyer bulbs, each containing around 20 ml medium. Use a batting top and tinfoil to cover the top of the Erlenmeyer bulb, and then put the bulbs with broth in the autoclave at 121 °C in 15min.

CFU plate, Marine Agar

55,1gram (Difco™ 2216, Becton, Dickinson and Company) of the powder in 1liter with dH_2O (distilled water), mix well and heat or boil for 1min while stir. Autoclave at 121 °C in 15min. Work in the ventilation cabinet when pouring out 20-25ml Marin Agar in each petri-dish, just enough to cover the bottom of the petri-dish. Put the lid on when the agar have stiffened.

7.2 Cultivation of the Vibrio

Growth medium, TSB, Tryptic Soy Broth

30gram (Bacto™ Tryptic Soy Broth, Soybean-Casein Digest, Medium, Becton, Dickinson and Company) of the powder in 1liter with dH_2O (distilled water or osmosis water), added 0,5% extra NaCl, mix well and heat slightly to completely dissolve the powder. Spread the broth in several 250ml Erlenmeyer bulbs, each containing around 100 ml medium. Use a batting top and tinfoil to cover the top of the Erlenmeyer bulb, and then put the bulbs with broth in the autoclave at 121 °C in 15min.

Agar plate, TSA, Tryptic Soy Agar

40gram (Difco™ Tryptic Soy Agar, Soybean-Casein Digest Agar, Becton, Dickinson and Company) of the powder in 1liter with dH_2O (distilled water or osmosis water), added 0,5% extra NaCl, mix thoroughly and heat with frequent agitation and boil for 1min to completely

dissolve the powder. Autoclave at 121°C in 15min. Work in the ventilation cabinet when pouring out 20-25ml Tryptic Soya Agar in each petri-dish, just enough to cover the bottom of the petri-dish. Put the lid on when the agar have stiffened.

7.3 Challenge dose protocol for Vibrio anguillarum/Listonella anguillarum performed by Kristian Dam at the University of Bergen

Cultivation

V. anguillarum cultured in 250ml Erlenmeyer bulbs holding 100ml Tryptone Soy Broth added 0,5% extra NaCl. Incubated at 60RPM and 20°C until the cultures reach about 0,7-0,8 in OD_{600} value (0,5-0,6 for HI610). Estimated generation time is 90min for NB10 types

Washing

1. 24ml growth culture is taken out at OD_{600} about 0,7 – 0,8. (For HI610 the OD_{600} values are 0,5 – 0,6)
2. It is centrifuged with 1825xg (2800RPM using an Allegra X-15R Centrifuge from Beckman coulter) at 20°C for 8min.
3. The supernatant is poured of, leaving a viscous pellet at the bottom of the centrifugal tube. 20ml of autoclaved and aerated 80% seawater at 20°C is transferred into the centrifugal tube using a pipette. The pellet is re-suspended by gentile shaking of the tube by hand.
4. Step nr. 2 and 3 are repeated twice, giving a total of three centrifugations. The final re-suspension of the pellet is done using a whirl-mixer.

Bacterial count

Counting chamber info: depth 0,02mm, square-net 0,0025mm^2, 1cell/large square equals 1,25x10^6 cells/ml, 1 cell/small square equals 20x10^6 cells/ml

1. About 20µl of stock-suspension is placed upon the countingchamber and a necessary dilution is evaluated.
2. The countingchamber is rinsed using 70% ethanol. Appropriate volume of stock suspension is taken out and diluted to suitable stock-suspensions in 8% formalin.
3. About 20µl diluted stock-suspension is placed on the countingchamber and is counted (I usually count all the bacteria within the chosen squares. I count the 4 large squares in each corner and the 4 large squares in the middle)
4. The counting error is rectified using: $n^{(1/2)}$, meaning that if I count 400 cells the counting error is 20 cells or 5%, these 20 cells are added to the outcome of the count.

5. The cell count should be performed at least 3 times for each bacterial suspension, this so that average, standard deviation and standard error can be calculated.
6. Average number of counted cells is multiplied with amount of cells/ml/large square specified for the counting chamber. This is then multiplied with the dilution used.

Now the bacterial concentration in the stock suspension is known

Making the challenge dose

1. Find all necessary details; well-volume, desired challenge dose concentration in the well, desired challenge dose volume to add to each well, and desired challenge dose stock volume.
2. The challenge dose is produced using this algorithm:

$$\mathbf{C1 \times V1 = C2 \times V2}$$

I want to find V1, which is the amount of stock-suspension that is to be transferred into V2 (prior to adding V1 into V2 an equal volume as V1 must be taken out of V2)

$$\mathbf{V1 = (C2 \times V2)/C1}$$

V2 = desired challenge dose stock

C1 = counted bacterial concentration

C2 = desired dose concentration within the well, this must be multiplied with the well-volume

V1 = volume stock suspension to transfer, this concentration must increase as much as the challenge dose volume is decreased.

3. The challenge dose suspension is finished

7.4 Information about the egg batches from hatchery A

Eggs for challenge experiment number 3

Incubator number: **220**

Generation: 2008
Buyer: Øivind Bergh
Age when transported: 44,1
Disinfection: No

Incubation date: 18.03.2011
Incubated: 600ml
Fertilisation / % OK cells: 54/82
Sediment out unfertilisated eggs before incubation

Date	Temp.	Day degrees	ml dead eggs	ml of eggs in incubator	total mortality in %	daily mortality in %
00.jan	3,5			600		
01.jan	3,8	7,3	35	565	5,8	5,8
02.jan	3,6	10,9	90	475	15,9	15,9
03.jan	4,0	14,9	37	438	22,5	7,8
04.jan	3,5	18,4	40	398	29,6	9,1
05.jan	3,3	21,7	30	368	34,9	7,5
06.jan	3,2	24,9		368	34,9	0,0
07.jan	3,1	28,0	55	313	44,6	14,9
08.jan	3,2	31,2		313	44,6	0,0
09.jan	3,3	34,5	15	298	47,3	4,8
10.jan	3,2	37,7		298	47,3	0,0
11.jan	3,2	40,9	3	295	47,8	1,0
12.jan	3,2	44,1		295	47,8	0,0
13.jan		44,1		295	47,8	0,0
14.jan		44,1		295	47,8	0,0
15.jan		44,1		295	47,8	0,0
16.jan		44,1		295	47,8	0,0
17.jan		44,1		295	47,8	0,0
18.jan		44,1		295	47,8	0,0
19.jan		44,1		295	47,8	0,0
20.jan		44,1		295	47,8	0,0
21.jan		44,1		295	47,8	0,0
22.jan		44,1		295	47,8	0,0
23.jan		44,1		295	47,8	0,0
24.jan		44,1		295	37,9	0,0

Egg for challenge experiment number four and five

Incubator number: **220**

Buyer: Øivind Bergh

Age group: 2008

Incubated: 01.04.2011

Amount (ml) egg incubated: 700

Age (day degrees) at 1.delivery, experiment no. 4: ca 45

Age (day degrees) at 2.delivery, experiment no. 5: ca 47,9

Fertilisation percent:82

% ok cells: 91

Disinfection: Buffodine

Sediment out unfertilisated egg before incubation: No at first delivery

Sediment out unfertilisated egg before incubation: Yes at second delivery

Date	Temp.	day degrees	ml dead eggs	ml egg in incubator	total mortality in %	dayli mortality in %
01.apr	3,5			700		
02.apr	3,5	7	145	555	20,7	
03.apr	3,5	10,5	25	530	4,5	4,5
04.apr	3,7	14,2	25	505	9,0	4,7
05.apr	3,7	17,9	35	470	15,3	6,9
06.apr	4,0	21,9	10	460	17,1	2,1
07.apr	3,7	25,6		460	17,1	0,0
08.apr	3,7	29,3	60	400	27,9	13,0
09.apr	3,8	33,1		400	27,9	0,0
10.apr	3,7	36,8	12	388	30,1	3,0
11.apr	3,7	40,5		388	30,1	0,0
12.apr	3,7	44,2		388	30,1	0,0
13.apr	3,7	47,9		388	30,1	0,0
14.apr		47,9		388	30,1	0,0
15.apr		47,9		388	30,1	0,0
16.apr		47,9		388	30,1	0,0
17.apr		47,9		388	30,1	0,0
18.apr		47,9		388	30,1	0,0
19.apr		47,9		388	30,1	0,0
20.apr		47,9		388	30,1	0,0
21.apr		47,9		388	30,1	0,0
22.apr		47,9		388	30,1	0,0
23.apr		47,9		388	30,1	0,0

7.5 Information about the egg batches from hatchery B

Eggs delivered for experiment number two:

Delivered: 6/12-10:

Fertilisation 28/11-10, Temperature 7,0 °C, Fertilisation % were 80

Eggs delivered for experiment number five:

Delivered 13/4-11:

Fertilisation 5/4-11, Temperature 7,0 °C, Fertilisation % were 85

7.6 Tables of the challenge experiment designs

Table 7.6. a): Table for challenge experiment number 1, only experiment at 7°C

Group no.	Bacteria 1	Dose bac 1	Bacteria 2	Dose bac 2	No. trays
1	27-4	High dose			3
1B	27-4	High dose	V. HI610	Low dose	3
1C	27-4	High dose	V.HI610 mm	Low dose	3
1D	27-4	High dose	V. GFP	Low dose	3
1E	27-4	High dose	V. mCherry	Low dose	3
2	M23-3.1	High dose			3
2B	M23-3.1	High dose	Vibrio	Low dose	3
3	M43-2.3	High dose			3
3B	M43-2.3	High dose	Vibrio	Low dose	3
4	F1926	High dose			3
4B	F1926	High dose	Vibrio	Low dose	3
5	JBB1001	High dose			3
5B	JBB1001	High dose	Vibrio	Low dose	3
6	Aq10	High dose			3
6B	Aq10	High dose	Vibrio	Low dose	3
7	Positive V. HI610	High dose			3
7B	Positive V. HI610	Low dose			3
8	V. HI610mm	High dose			3
8B	V. HI610mm	Low dose			3
9	Negative control				3
					60

Table 7.6 b): Table for challenge experiment number 2

Temp.	Gr.no.	Bacteria 1	Dose bac 1	Bacteria 2	Dose bac 2	No. trays
7°C	1	Negative control				3
7°C	2	Positive con. V.HI610	High dose			3
7°C	3	27-4	High dose			3
7°C	4	27-4	High dose	V. HI610	High dose	3
7°C	5	27-4 first	High dose	V.HI610 48h	High dose	3
7°C	6	V. HI610 first	High dose	27-4 48h	High dose	3
7°C	7	M23-3.1	High dose			3
7°C	8	M23-3.1	High dose	V.HI610	High dose	3
7°C	9	M23-3.1 first	High dose	V.HI610 48h	High dose	3
7°C	10	V. HI610 first	High dose	M23-3.1 48h	High dose	3
15°C	1B	Negative control				3
15°C	2B	Positive con. V.HI610	High dose			3
15°C	3B	27-4	High dose			3
15°C	4B	27-4	High dose	V. HI610	High dose	3
15°C	5B	27-4 first	High dose	V.HI610 48h	High dose	3
15°C	6B	V. HI610 first	High dose	27-4 48h	High dose	3
15°C	7B	M23-3.1	High dose			3
15°C	8B	M23-3.1	High dose	V.HI610	High dose	3
15°C	9B	M23-3.1 first	High dose	V.HI610 48h	High dose	3
15°C	10B	V. HI610 first	High dose	M23-3.1 48h	High dose	3
						64

Table 7.6 c): Table for challenge experiment number 3, 4 & 5

Temp.	Gr.no.	Bacteria 1	Dose bac.1	Bacteria 2	Dose bac.2	No. trays
7°C	1	Negative control				3
7°C	2	Positive con. V.HI610	High dose			3
7°C	3	27-4	High dose			3
7°C	4	27-4	High dose	V. HI610	High dose	3
7°C	5	27-4 first	High dose	V. HI610 48h	High dose	3
7°C	6	V. HI610 first	High dose	27-4 48h	High dose	3
7°C	7	M23-3.1	High dose			3
7°C	8	M23-3.1	High dose	V.HI610	High dose	3
7°C	9	M23-3.1 first	High dose	V. HI610 48h	High dose	3
7°C	10	V. HI610 first	High dose	M23-3.1 48h	High dose	3
7°C	11	P.g wt	High dose			3
7°C	12	P.g wt	High dose	V. HI610	High dose	3
7°C	13	P.g wt first	High dose	V.HI610 48h	High dose	3
7°C	14	V. HI610 first	High dose	P.g wt 48h	High dose	3
7°C	15	P.g Pda8	High dose			3
7°C	16	P.g Pda8	High dose	V.HI610	High dose	3
7°C	17	P.g Pda8 first	High dose	V.HI610 48h	High dose	3
7°C	18	V. HI610 first	High dose	P.g Pda8	High dose	3
15°C	1B	Negative control				3
15°C	2B	Positive con. V.HI610	High dose			3
15°C	3B	27-4	High dose			3
15°C	4B	27-4	High dose	V. HI610	High dose	3
15°C	5B	27-4 first	High dose	V.HI610 48h	High dose	3
15°C	6B	V. HI610 first	High dose	27-4 48h	High dose	3
15°C	7B	M23-3.1	High dose			3
15°C	8B	M23-3.1	High dose	V.HI610	High dose	3
15°C	9B	M23-3.1 first	High dose	V.HI610 48h	High dose	3
15°C	10B	V. HI610 first	High dose	M23-3.1 48h	High dose	3
15°C	11B	P.g wt	High dose			3
15°C	12B	P.g wt	High dose	V.HI610	High dose	3
15°C	13B	P.g wt first	High dose	V.HI610 48h	High dose	3
15°C	14B	V. HI610 first	High dose	P.g wt 48h	High dose	3
15°C	15B	P.g Pda8	High dose			3
15°C	16B	P.g Pda8	High dose	V.HI610	High dose	3
15°C	17B	P.g Pda8 first	High dose	V.HI610 48h	High dose	3
15°C	18B	V. HI610 first	High dose	P.g Pda8	High dose	3
						108

7.7 Example of statistical matrixes

Example of statistical matrixes used in R, examples from Challenge experiment 5, 7°C.

```
#Challenge experiment 5 7°C mortality 10dph#

#PC vs. NC
mortality <- matrix(c(72, 0, 8, 64), ncol=2)
chisq.test(mortality)

#PC vs. only 27-4
mortality <- matrix(c(72, 0, 6, 66), ncol=2)
chisq.test(mortality)

#PC vs. 27-4 and HI610
mortality <- matrix(c(72, 0, 68, 4), ncol=2)
chisq.test(mortality)

#PC vs. 27-4first and HI610 48h after
mortality <- matrix(c(72, 0, 11, 61), ncol=2)
chisq.test(mortality)

#PC vs. HI610first and 27-4 48h after
mortality <- matrix(c(72, 0, 72, 0), ncol=2)
chisq.test(mortality)

#PC vs. only M23-3.1
mortality <- matrix(c(72, 0, 8, 64), ncol=2)
chisq.test(mortality)

#PC vs. M23-3.1 and HI610
mortality <- matrix(c(72, 0, 5, 67), ncol=2)
chisq.test(mortality)

#PC vs. M23-3.1first and HI610 48h after
mortality <- matrix(c(72, 0, 9, 63), ncol=2)
chisq.test(mortality)

#PC vs. HI610first and M23-3.1 48h after
mortality <- matrix(c(72, 0, 63, 9), ncol=2)
chisq.test(mortality)
```

7.8 Tables of the p-values for experiment 3, 4 and 5 at 7°C and 13°C

Table 7.8 a): temperature 7°C

Strain	challenge experiment no. 3 10 pdh				challenge experiment no. 4 10 pdh				challenge experiment no. 5 10 pdh			
	C.m. (%)	C.m. (%)	χ^2	**p-value**	C.m. (%)	C.m. (%)	χ^2	**p-value**	C.m. (%)	C.m. (%)	χ^2	**p-value**
PC vs. NC	98,61	30,56	69,95	**$2{,}2 \times 10^{-6}$**	100	44,44	52,65	**$3{,}98 \times 10^{-13}$**	100	11,11	111,63	**$2{,}2 \times 10^{-16}$**
PC vs. only 27-4	98,61	12,50	104,7	**$2{,}2 \times 10^{-6}$**	100	27,78	78,29	**$2{,}2 \times 10^{-16}$**	100	8,33	118,18	**$2{,}2 \times 10^{-16}$**
PC vs. 27-4 and HI610	98,61	63,89	26,26	**$2{,}99 \times 10^{-7}$**	100	88,89	-	-	100	94,44	-	-
PC vs. 27-4first and HI610 48h after	98,61	50,00	42,05	**$8{,}91 \times 10^{-11}$**	100	23,61	85,78	**$2{,}2 \times 10^{-16}$**	100	15,28	102,39	**$2{,}2 \times 10^{-16}$**
PC vs. HI610first and 27-4 48h after	98,61	97,22	-	-	100	97,22	-	-	100	100	Inf	-
PC vs. only M23-3.1	98,61	19,44	90,05	**$2{,}2 \times 10^{-6}$**	100	11,11	111,6	**$2{,}2 \times 10^{-16}$**	100	11,11	111,63	**$2{,}2 \times 10^{-16}$**
PC vs. M23-3.1 and HI610	98,61	16,67	95,68	**$2{,}2 \times 10^{-6}$**	100	9,72	114,9	**$2{,}2 \times 10^{-16}$**	100	6,94	121,59	**$2{,}2 \times 10^{-16}$**
PC vs. M23-3.1first and HI610 48h after	98,61	13,89	101,59	**$2{,}2 \times 10^{-6}$**	100	12,50	108,5	**$2{,}2 \times 10^{-16}$**	100	12,50	108,47	**$2{,}2 \times 10^{-16}$**
PC vs. HI610first and M23-3.1 48h after	98,61	16,67	95,68	**$2{,}2 \times 10^{-6}$**	100	19,44	93,79	**$2{,}2 \times 10^{-16}$**	100	87,50	-	-
PC vs. only P.g wt	98,61	11,11	107,80	**$2{,}2 \times 10^{-6}$**	100	9,72	114,9	**$2{,}2 \times 10^{-16}$**	100	8,33	118,18	**$2{,}2 \times 10^{-16}$**
PC vs. P.g wt and HI610	98,61	13,89	101,59	**$2{,}2 \times 10^{-6}$**	100	11,11	111,6	**$2{,}2 \times 10^{-16}$**	100	40,28	58,49	**$2{,}1 \times 10^{-14}$**
PC vs. P.g wt first and HI610 48h after	98,61	16,67	95,68	**$2{,}2 \times 10^{-6}$**	100	13,89	105,4	**$2{,}2 \times 10^{-16}$**	100	22,22	88,39	**$2{,}2 \times 10^{-16}$**
PC vs. HI610first and P.g wt 48h after	98,61	73,61	16,78	**$4{,}196 \times 10^{-5}$**	100	69,44	23,66	**$1{,}15 \times 10^{-6}$**	100	98,61	0	1
PC vs. only P.g Pda8	98,61	13,89	101,59	**$2{,}2 \times 10^{-6}$**	100	16,67	99,46	**$2{,}2 \times 10^{-16}$**	100	13,89	105,39	**$2{,}2 \times 10^{-16}$**
PC vs. P.g Pda8 and HI610	98,61	47,22	45,57	**$1{,}47 \times 10^{-11}$**	100	90,28	-	-	100	95,83	-	-
PC vs. P.g Pda8 first and HI610 48h after	98,61	40,28	55,01	**$1{,}196 \times 10^{-13}$**	100	15,28	102,4	**$2{,}2 \times 10^{-16}$**	100	29,17	75,90	**$2{,}2 \times 10^{-16}$**
PC vs. HI610first and P.g Pda8 48h after	98,61	100	-	-	100	100	Inf	-	100	100	Inf	-

Table 7.8 b): temperature 13°C

Strain	challenge experiment no. 4 8 pdh				challenge experiment no. 5 8 pdh			
	C.m. %	C.m. %	χ^2	**p-value**	C.m. %	C.m. %	χ^2	**p-value**
PC vs. NC	87,50	48,61	23,29	**$1{,}40 \times 10^{-6}$**	100	20,83	91,06	**$2{,}2 \times 10^{-16}$**
PC vs. only 27-4	87,50	41,67	31,09	**$2{,}46 \times 10^{-8}$**	100	22,22	88,39	**$2{,}2 \times 10^{-16}$**
PC vs. 27-4 and HI610	87,50	47,22	24,76	**$6{,}48 \times 10^{-7}$**	100	100	-	-
PC vs. 27-4first and HI610 48h after	87,50	58,33	14,07	**0,00018**	100	25,00	83,23	**$2{,}2 \times 10^{-16}$**
PC vs. HI610first and 27-4 48h after	87,50	86,11	0	1	100	100	-	-
PC vs. only M23-3.1	87,50	31,94	43,91	**$3{,}44 \times 10^{-11}$**	100	19,44	93,79	**$2{,}2 \times 10^{-16}$**
PC vs. M23-3.1 and HI610	87,50	44,44	27,84	**$1{,}317 \times 10^{-7}$**	100	88,89	-	-
PC vs. M23-3.1first and HI610 48h after	87,50	45,83	26,28	**$2{,}95 \times 10^{-7}$**	100	5,56	125,1	**$2{,}2 \times 10^{-16}$**
PC vs. HI610first and M23-3.1 48h after	87,50	55,56	16,50	**$4{,}86 \times 10^{-5}$**	100	97,22	-	-
PC vs. only P.g wt	87,50	43,06	29,44	**$5{,}76 \times 10^{-8}$**	100	11,11	111,6	**$2{,}2 \times 10^{-16}$**
PC vs. P.g wt and HI610	87,50	45,83	26,28	**$2{,}95 \times 10^{-7}$**	100	91,67	-	-
PC vs. P.g wt first and HI610 48h after	87,50	50,00	21,85	**$2{,}95 \times 10^{-7}$**	100	16,67	99,46	**$2{,}2 \times 10^{-16}$**
PC vs. HI610first and P.g wt 48h after	87,50	65,28	8,66	**0,0032**	100	100	-	-
PC vs. only P.g Pda8	87,50	45,83	26,28	**$2{,}95 \times 10^{-7}$**	100	12,50	108,5	**$2{,}2 \times 10^{-16}$**
PC vs. P.g Pda8 and HI610	87,50	70,83	5,095	**0,024**	100	98,61	-	-
PC vs. P.g Pda8 first and HI610 48h after	87,50	54,17	17,78	**$2{,}48 \times 10^{-5}$**	100	19,44	93,79	**$2{,}2 \times 10^{-16}$**
PC vs. HI610first and P.g Pda8 48h after	87,50	98,61	5,27	**0,022**	100	98,61	-	-