

UNIVERSIDADE DO ALGARVE

Faculdade de Ciências do Mar e do Ambiente

**Development of a Gnotobiotic Growth System for
Turbot (*Psetta maxima*)**

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Mestrado em Aquacultura e Pescas

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SUMMARY

Turbot (*Psetta maxima*), is one of the most important marine fin fishes for aquaculture in Northern Europe because of its relative rapid grow and high market value. However, disease outbreaks are being recognized as a significant constraint to aquaculture production and evidence points towards bacteria as a major cause of the problem. Antibiotics have been applied in aquaculture production to control diseases, but the malpractice is increasing bacteria resistance, reducing the efficacy of fish disease control, transferring resistance to human pathogens, as well as adverse ecological impacts. The use of probiotics provides an ecological alternative to the use of antimicrobial compounds, for disease prevention in aquaculture.

The aim of this work was to develop a methodology to culture turbot under gnotobiotic conditions using glutaraldehyde, ampicillin, rifampicin, tetracycline, and kanamycin as well as evaluate the effect of the LVS3-RP4 transconjugant (*Aeromonas hydrophila*-LVS3 and *Escherichia coli*-RP4) as a probiont, in these treatments.

Two experiments were done using turbot eggs and larvae in a starvation experiment. The experiments were conducted at 16°C and the eggs were previously treated with glutaraldehyde 200 mg/l [50% (v/v) in water] for 5 minutes, afterwards different combinations of antibiotics and the application of 10⁵ CFU/ml of LVS3-RP4 transconjugant were added. The disinfection efficiency and hatchability of the turbot eggs and the survival of the larvae were evaluated. Experiments were conducted to check the LVS3-RP4 transconjugant development within the treatments.

The egg quality has influenced the experiment results on hatching and survival. The LVS3-RP4 transconjugant has demonstrated its probiotic effect in the larvae survival experiment although it entered a viable but non-culturable (VBNC) state.

It became also clear that only using glutaraldehyde for egg surface disinfection can lead to a lower larvae survival, because this treatment probably kills the majority of the bacteria unbalancing the natural flora from the egg surface, allowing the opportunistic pathogens to grow. Subsequently, through the transference of bacteria from the egg surface to the hatched larvae, some reminiscent opportunistic bacteria grew and caused adverse effects on larvae.

The subsequent use of ampicillin also was not efficient probably because of the large number of bacteria resistant to this antibiotic.

SAMENVATTING

Tarbot (*Psetta maxima*) is één van de belangrijke mariene vissen voor de aquacultuur in Noord-Europa omwille van zijn relatief hoge groeisnelheid en hoge marktwaarde.

Ziektes worden echter erkend als belangrijke hinderpalen in de aquacultuurproductie en bacteriën werden aangetoond als de belangrijkste oorzaak van dit probleem. Antibiotica werden aangewend in aquacultuurproductie om ziektes te bestrijden, maar door verkeerdelijk gebruik stijgt de bacterie resistentie, waardoor de efficiëntie van de controle van visziektes daalt, de resistentie wordt overgedragen naar menselijke pathogenen en daarnaast is er de negatieve ecologische impact. Het gebruik van probiotica voorziet in een ecologisch alternatief voor het gebruik van antibiotica in het voorkomen van ziektes in aquacultuur.

Het doel van deze studie was zowel het ontwikkelen van een methodologie om tarbot onder gnotobiotische condities te houden, gebruikmakend van glutaraaldehyde, ampicilline, rifampicine, tetracycline en kanamycine, evenals het effect van de LVS3-RP4 transconjugant (*Aeromonas hydrophila*; LVS3 and *Escherichia coli*; RP4) als probiont in deze behandelingen te evalueren.

Er werden 2 experimenten uitgevoerd waarbij tarboteieren en –larven werden gebruikt in een starvatie-experiment. De experimenten werden uitgevoerd bij 16°C. De eieren werden eerst behandeld met glutaraldehyde (200 mg/l, 50% actief bestanddeel) voor 5 minuten. Daarna werden verschillende antibiotica combinaties en de transconjugant LVS3-RP4 (10^5 KVE/ml) toegevoegd. De desinfectie efficiëntie en het ontluikingspercentage van de tarboteieren werd geëvalueerd naast de overleving van de larven.

Er werden ook experimenten uitgevoerd om de ontwikkeling van de LVS3-RP4 transconjugant te volgen in de verschillende behandelingen.

De eikwaliteit heeft de resultaten van de experimenten beïnvloed, zowel het ontluikingspercentage als de overleving. Het probiotisch effect van de LVS3-RP4 transconjugant werd aangetoond in het starvatie-experiment ondanks het feit dat de probiont in een ‘levende-maar-niet-kweekbare’ toestand verkeerde.

Het werd ook duidelijk dat het gebruik van glutaraldehyde voor het desinfecteren van het ei-oppervlak alleen, kan leiden tot een lager ontluikingspercentage, omdat deze behandeling waarschijnlijk de meerderheid van de bacteriën doodt waardoor een onevenwicht in de natuurlijke flora van het ei-oppervlak wordt veroorzaakt wat de groei van opportunistische pathogenen toelaat. Daaropvolgend koloniseerden de bacteriën van het ei-oppervlak de pasontloken vislarven, waardoor ze negatieve effecten veroorzaakten bij de larven.

Het werd ook duidelijk dat toevoeging van ampicilline tijdens de incubatie van de eieren geen positief effect had, omdat waarschijnlijk reeds veel bacteriën resistent zijn voor dit antibioticum.

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1 INTRODUCTION

Aquaculture, the husbandry of aquatic animals and plants, has been practiced since the earliest records of human history and is a rapidly growing industry in many parts of the world. In comparison, the output from capture fisheries has actually fallen because the intensive fishing efforts that affects the resource base. Overfishing and depletion of stocks have become a living reality and the need to enhance or create new stocks by human intervention has begun to be recognized (U.S. National Research Council, 1992; OECD, 2003).

The global production from capture fisheries and aquaculture supplied about 101 million tonnes of food fish in 2002, providing an apparent per capita supply of 16.2 kg. The world marine capture fisheries production decreased to about 84 million tonnes in 2001 and remained at this level in 2002, after a production of 87 million tonnes in 2000. Inland capture fisheries production has a small fluctuation during 2000–02 (FAO, 2004).

As world population continues to expand, with current projections being for an increase from 6 billion people in the year 2000 to 9-10 billion by 2050, fisheries products are one of the many food groups that will come under increasing pressure. Almost all the world's natural fisheries resources are fully exploited or already over exploited, and the challenge for aquaculture is to expand in order to satisfy the fish supplies in a sustainable way providing economic development to the local populations (UN Atlas of the Oceans, 2001).

One of the most valuable fish of the north-eastern Atlantic is the Turbot, *Psetta maxima*. The potential of this flatfish for farming was recognised during the early 1970s when the turbot appeared to offer the best prospect for commercial exploitation because of its relatively rapid growth rate and high value (Moksness *et al*, 2004).

Disease outbreaks are among the biggest problems related with aquaculture development and are being increasingly recognized as a significant constraint to

production and trade, affecting economic development of the sector in many countries of the world (Subasinghe, 1997).

Poor performance on fish could be caused by toxic substances, improper nutrition, poor water quality, physical damage or genetics; or by infectious diseases caused by pathogenic organisms (parasites, bacteria, virus and fungi). These latter are contagious and represent the most important diseases in aquaculture, because they increase the cost production due to the losses in dead fish, costs of treatments or decreased growth rate of diseased and convalescent fish. Outbreaks of disease are typically caused by widely distributed, opportunistic pathogens. In natural systems, despite the main presence of opportunistic pathogen, infection incidence is apparently low, but in finfish cultures or shellfish hatcheries where stresses lower resistance and stocking density facilitates transmission of disease, impacts of disease outbreaks can be severe (Johnston and Santillo, 2002).

Infectious diseases are typically controlled by eradication of the pathogen through treatment with antibiotics or chemotherapeutics, and/or by preventative measures such as the use of probiotics or vaccines.

Antibacterial chemotherapy has been applied in aquaculture, to control diseases, for over 50 years, with early attempts to use sulphonamides in the treatment of furunculosis in trout and the tetracyclines against a range of Gram-negative pathogens. However, they did not come into general use until the 1970s when the sulphonamides were used. Since then, their use has grown both in numbers and quantity, as the problem of bacterial disease has increased (Weston, 2000).

As a consequence of the malpractice of the antimicrobials an increase in the drug resistance among bacterial fish pathogens has been reported during the last years. These resistances reduce the efficacy of fish disease control, but it also could contribute to their transmission to human pathogens. The accumulation of residues in fish meat is another problem derived from the use of antimicrobials in fish farms (Blanco, 2000).

The alternative to the antimicrobials for controlling infectious diseases is vaccination. This practice in fish is becoming an increasingly important part of aquaculture, aimed at preventing and controlling infectious diseases in fish farms and at

reducing the use of antimicrobial drugs. In the recent years, fish vaccination has greatly benefited from the better understanding of the immune system of fish, and from the identification of the virulence factors of the main fish pathogens (Gudding *et al.*, 1999). Although, live, attenuated vaccines for some authorities are considered to be undesirable because of the risk of uncontrolled spread in the aquatic environment. Vaccines are not available for all pathogens, usually involve stressful handling of animals, and are ineffective with most invertebrates and early life stages of vertebrates (Olafsen, 2001).

The use of probiotics for disease prevention and improved nutrition in aquaculture is becoming increasingly popular due to an increasing demand for healthy environment aquaculture. Probiotics for aquatic organisms have been defined as microbial cells that are administered in such a way as to enter the gastrointestinal tract where they help in improving health (Gatesoupe, 1999).

The need to develop effective alternative solutions to replace antibiotics in aquaculture are obviously urgent as well as a better understanding of the recent techniques applied to mitigate the diseases outbreaks.

Performing experiment with fish larvae in the presence of well characterised microbial community (gnotobiotic systems) can be very useful to increase the understanding of the mechanisms involved in host-microbe interactions. Hopefully the knowledge acquired in this way, can be implemented in non-gnotobiotic conditions.

The main objectives of the experiments were: develop a methodology to culture turbot under gnotobiotic conditions; to evaluate the effect of treatments using glutaraldehyde, ampicillin, rifampicin, tetracycline, and kanamycin; and evaluate the effect of the LVS3-RP4 transconjugant as a probiont, in these treatments.

2 LITERATURE REVIEW

2.1. TURBOT

2.1.1. Systematic

The turbot was named *Psetta maxima* by Linnaeus (1758) and secondly by Rafinesque in 1810, as *Scophthalmus maximus*. The European turbot is a large teleost flatfish highly regarded for its excellent taste and is one of the most expensive marine fish (Nashand and Novotny, 1995).

The turbot are circular in shape, with both eyes on the upper side of the head, and large fan shaped flattened tail as can be seen in Fig.1. They are normally dark brown to black on the dorsal side and white on the underside. Small lumps (termed tubercules) are present on the dorsal side, which are believed to act as sensory organs, when they are buried in the sand (Stickney, 2000).

Phylum: Chordata

Superclass: Gnathostomata

Class: Teleostomi

Subclass: Neopterygii

Order: Pleuronectiformes

Family: Scophthalmidae

Genus: Scophthalmus

Species: *Scophthalmus maximus*

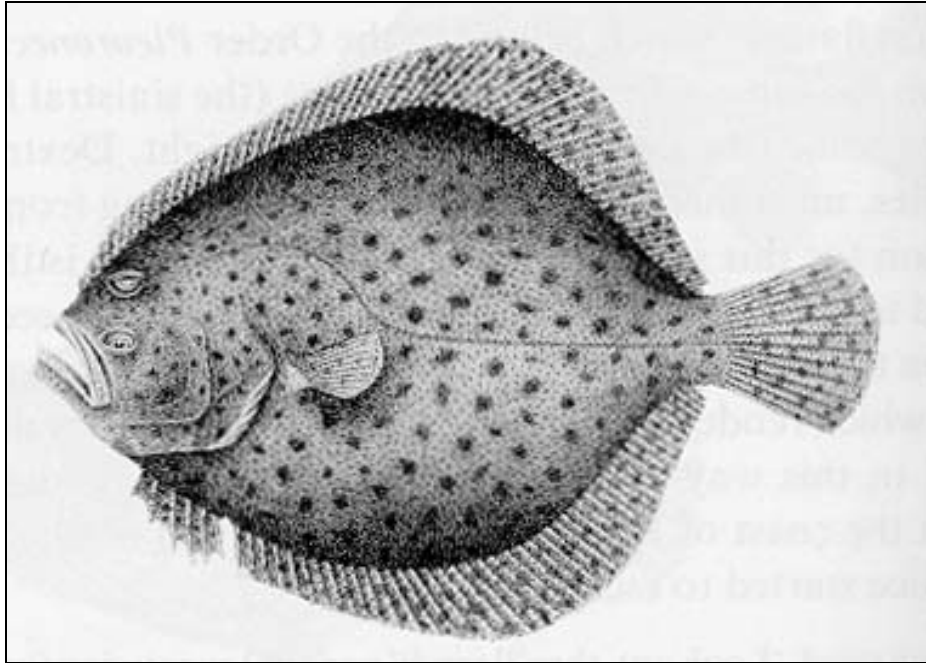


Fig. 1 Turbot (*Psetta maxima*)

Key to the flatfishes (Pleuronectiformes) of Europe (Muus and Nielsen, 1999).

1 a	Upper jaw protruding, mouth curved. Rear end of front gill cover covered by skin (soles).	2	
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17 b	No mucus pores or anal spine; body fleshy and oval-shaped.	-	<i>Microstomus kitt</i>

2.2. BIOLOGY

2.2.1. Geographical distribution and habitat

The turbot range extends from the Norwegian coast through the North Sea and Baltic to the Mediterranean. A separate species of turbot (*Scophthalmus maeoticus*) is found in the Black Sea (Nash and Novotny, 1995).

The turbot, in their first year, prefer shallow water on exposed sandy beaches where there is heavy wave action. In the second year all individuals begin to move into deeper water, but still retain their substrate preferences (Pitcher and Hart, 1982).

2.2.2. Diet

The turbot regime during the larval phase includes planktonic species such as copepod nauplii and others small crustaceans (Jones, 1972). Mature turbot are predatory fish, mostly preying on the juveniles of both commercial valuable and invaluable fish species. A study found the higher filling indices in the turbot intestinal tract in September, which revealed that turbot, was feeding most intensively in this month (Stankus, 2000).

2.2.3. Reproduction and life cycle

The wild turbot, when become sexually mature tend to come closer to shore to spawn around August as water increases the temperature about 14-16°C. The female can produce up to three million eggs over a spawning period of 5-8 weeks and depending on the surrounding bottom habitat the dorsal side can change from dark to light brown in the same period. Male and female reach sexual maturity in about four to five years, weighing on average 3-4 kg at spawning. The fertilized eggs stay within the water column and the success of the external fertilization depends on the tides and currents (Stickney, 2000). The turbot eggs are small and pelagic, with a single oil globe. Considerable variation on egg size is encountered between females, but all eggs tend to

lie within the range 0.9 to 1.2 mm in diameter. When the eggs are fertilized, they are transparent and buoyant, while dead eggs become opaque and sink (Jones, 1972).

The pelagic period of the eggs takes about 60 days at temperatures around 16°C. Turbot has an air bladder in the pelagic larval phase and after hatching the larvae are carried out for shallow water by currents (Jones, 1972).

The metamorphosis starts on turbot about day 25-30 after hatching, to the flattened shape, with the left eye migration to the upper side. At this stage the turbot will also start to descend to the benthonic environment to live as a benthic organism (Stickney, 2000). The adult fish, migrate to deeper waters about 20 to 70 meters (Jones, 1972). This preference of adult turbot for deeper and cooler water compared with juvenile turbot in ocean waters was in accordance with Imstrand *et al.* (1996) study, where the authors found a downshift in temperature optimum with size.

2.3. TURBOT CULTURE

For more than 10 years, turbot hatchery production has been a successful economic activity in Europe, but high technical risks still remain. Turbot survival is still rather low during the turbot hatchery phase and shows variability in hatchery success, ranging from close to 0% up to 50% survival among batches (Leclercq, 1994).

Survival rates of larval fish are highly variable and unpredictable under intensive rearing conditions because the larvae are hatched into the same ecosystem as potential bacterial pathogens (Lein *et al.*, 1992). In general, poor survival, growth and quality of the larvae can be explained by microbial conditions during egg and yolk sac stages (Salvesen *et al.*, 1991).

2.3.1. Larvae development and rearing

Turbot ovulate in captivity and produce good quality at temperatures in the range 10-14°C and, with manipulation of the photoperiod, maturation can be induced at any time of the year (Pillay, 1990).

The turbot natural spawning is inhibited in culture tanks, unlike other flatfish like sole and plaice, but the eggs can be obtained by removing the fish from their tanks and hand-stripping, after artificial fertilization. The broodstock can be induced to spawn at different times of year by manipulation of artificial daylight to give an all-year-round supply of juveniles. The viability of eggs and larvae obtained in this way varies considerably, which to a large extent is due to the rapid deterioration in the quality of the eggs after ovulation (Moksness *et al.*, 2004; Smith, 1992; Stickney, 2000).

Female turbot are extremely fecund and can produce 1 million eggs per kg body weight. The eggs are generally incubated in up-welling systems stocked at a rate of between 2-3000 l⁻¹ (Moksness *et al.*, 2004).

According to Smith (1992), stripped eggs are fertilized and incubated for about six days before hatching. The hatched larvae live on their yolk sacs for two days and then start a critical period where the larvae require their first added nutrition. The length of turbot larvae on hatching is about 2.7 – 3.0 mm. During the yolk absorption the larvae are very inactive and lie at the surface. The eye becomes more densely pigmented few days after hatching, the mouth develops and the pectorals fins become functional (Jones, 1972).

The time at which food is first offered to the larvae influences subsequent survival rates. Also the nutritional value of the live prey organisms is strictly related with the survival (Jones, 1972; Merchie *et al.*, 1996). The larvae are reared in the hatchery initially on rotifers *Brachionus plicatilis* and with *Artemia* from around day 15 after hatching, and by day 35-40 they are weaned onto artificial diets. The live foods are enriched with appropriate algae that provide certain polyunsaturated fatty acids that are known to be essential dietary requirement for turbot and other marine species as well as the inert diets to ensure that they are of a suitable nutritional value. In all, it takes some 4-5 months to produce a turbot juvenile of 5 g ready to move to the on-growing phase (Moksness, 2004).

Poor egg quality and larvae mass mortality are prevalent in the intensive farming (Hansen and Olafsen, 1989). In the beginning of the larval period, the turbot depends largely on the availability of essential nutrients, the endogenous provision of these

nutrients through the egg stages, and possibly parental diet, might be an important tool in improving hatchery output (Lavens *et al.*, 1999).

Research on the dietary essential fatty acid requirements of marine fish larvae has considered the interactions of all three EFAs, docosahexaenoic acid (DHA, 22:6n-3), eicosapentaenoic acid (EPA, 20:5n-3) and arachidonic acid (ARA, 20:4n-6) (Sargent *et al.*, 1999). Turbot require a dietary supply of 20:5 or 22:6n-3 fatty acids, since they cannot synthesize these fatty acids from C18 precursors in significant amounts (Léger *et al.*, 1979). According to Castell *et al.* (1994), arachidonic acid has been shown to be essential in the growth and development of juvenile turbot.

Turbot larvae can be reared under stagnant or open-system conditions, without significant effect on growth rates by the different rearing systems (Şahin and Üstündağ, 2003). Circular indoor tanks up to 2.7m³ in size, supplied with warm water and continuous high-intensity of illumination (up to 3000 lux), have been successfully used for spawning and larval rearing. Newly hatched larvae reared at densities of 30-45 larvae per liter, in 60-450 liter tanks. Temperature between 18 and 20°C is maintained with 90 per cent water exchange/per day (Pillay, 1990).

2.3.2 Ongrowing

The preferred system for turbot ongrowing is land based tanks supplied with pumped sea water. The turbot are grown in tanks and raceways mainly constructed of concrete and usually covered to reduce light intensities and control algae growth which can result in tank fouling problems. Water depth is typically around 1 m. Tank area required in turbot farming should be in the range of 25-30 m² per tonne of output. Dry pelleted feeds for turbot are available and average conversion rates over the growout cycle of 1.5:1 can be achieved on a routine basis. Spanish producers still favour the use of moist pellets for fish of more than 200 g in weight. These diets compare favourably in cost with dry pellets although conversion rates are somewhat higher in the region of 1.9:1. The moist formulated feed has some disadvantages like the higher waste production and consequent water pollution, and the cost for storage (frozen) and handling. Water requirements can vary considerably depending on factors such as

temperature and whether or not the farms use pure oxygen. Therefore, farm water use can vary from 10 m³ to 30m³/h/t of stock held (Nash and Novotny, 1995).

2.4. TURBOT STATUS AND NUMBERS

The turbot is among the most valuable fish in the north-eastern Atlantic and it occurs from western Norway to southern Spain and throughout the Mediterranean. The European landings, which have fluctuated between 3.4 and 9 thousands tonnes over the last 30 years, are almost entirely generated as a by-catch of targeting other species (Moksness *et al.*, 2004).

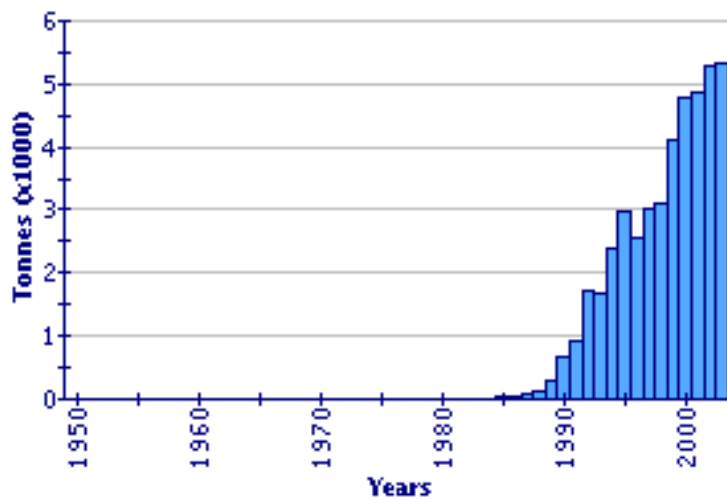


Fig. 2 Global aquaculture production of *Psetta maxima* (FAO, 2005)

The potential of turbot for farming was recognised and the Fig. 2 shown the increasing aquaculture production of *Psetta maxima* in a global scale. The French production jumped from 150 tonnes in 1993 to 980 tonnes in 1997. In 2002, the production stayed around 728 tonnes. The production from Portugal seems to fluctuate around 350-390 tonnes per year. Spain is the biggest producer of turbot in Europe, and the annual production of this country has nearly doubled since 1998. Spanish turbot production in 2002 was 3847 tonnes (75.9% of the global total). The turbot world production in 2002 was valued at US\$ 41.38 million (FAO, 2005).

2.5. MICROBIAL ASPECTS

Aquatic farmed animals are surrounded by an environment that supports their pathogens independently of the host animals providing high densities of opportunistic pathogens around the animal (Moriarty, 1998). Over the last four decades, many epizootics causing natural mortalities of fish have been attributed to infectious pathogens (Hansen and Olafsen, 1999). Various forms of interactions between bacteria and the hosts may occur during the intensive hatching of eggs and rearing of marine larvae. After transference to incubators, the eggs become heavily overgrown with bacteria that differ in number and characteristics from that in the sea (Olafsen, 2001). According to Reitan *et al.* (1998), fish larvae drink water at an early developmental stage, allowing the opportunistic pathogenic bacteria to colonize the gut of larvae fish before active feeding commences. A clear succession of bacteria phenotypes was found by Blanch *et al.* (1997), from day 1 to 90 after hatching. Oxidative Gram-negative rods were predominant at the initial stages followed by *Vibrio*, when highest mortalities of turbot larvae were observed. Evidencing the problem with opportunist pathogens, Munro *et al.* (1995) noticed low mortality of turbot larvae when cultured in absence of bacteria.

Disinfection of eggs is a positive factor for control of growth and transfer of bacteria in larviculture. For marine fish eggs, glutaraldehyde has suitable properties as a disinfectant, which improves hatching, development and survival of larvae (Skjermo and Vadstein, 1999).

2.5.1. Microbial control techniques

According to Davenport *et al.* (2003), in aquaculture, for pest control, treatment of disease, disinfection, anaesthesia and other functions a big variety of chemicals including biocides, antibiotics, therapeutics and hormones are used. All these substances represent a potential threat to the health of the cultured organisms, the indigenous biota and the human consumer. The aquaculture development is causing concern about the environmental impact of this industry on freshwater and marine ecosystems in many countries throughout the world.

Antibiotics can affect sedimentary biogeochemical processes, presumably by interference with bacterial ecology. Seven days after application of oxytetracycline, oxolinic acid, or flumequine to sediments in experimental tanks, sulphate reduction rates were reduced to less than 10% of the rates found in controls. This effect lasted at least 29 days, but was absent after 70 days (Davenport *et al.*, 2003).

Some antibiotics can present teratogen effects which produces birth defects. Common types of effects caused by teratogens on developing organisms include structural malformations, growth retardation, and death (Stine, 1996). The adverse effects of tetracycline in humans could be the inhibition of bone growth, maternal liver toxicity, and congenital defects (Gomella *et al.*, 1999).

2.5.2. Bacteria resistance

Bacteria can be innately resistant or may acquire resistance to antibiotics. Both types of resistance are probably equally important in the context of the treatment of infections. Acquired drug resistance may develop as a result of antibiotic-induced mutations altering the target site for the antibiotic, or by acquisition of new drug resistance genes from either the same or different species. The acquisition of resistance genes is mediated by transferable extrachromosomal genetic elements called plasmids, or special genetic elements known as transposons and integrons, which have the ability to integrate with both the chromosomes and plasmids (Rao, 1998).

Resistance is a description of the relative insusceptibility of a microorganism to a particular treatment under a particular set of conditions. There is a change in susceptibility that renders an agent ineffective against a certain organism and this organism is referred to as resistant (Krummerer, 2004). Antibiotics have a long history of mal-use in human health, agriculture and aquaculture and now the consequences are known. The problems related about the antibiotics, increased the research for probiotics as an alternative for disease prevention and control.

2.5.3. Probiotics as alternative

The probiotics have an important role in disease control strategies for aquaculture, and may provide an ecological alternative to the use of antimicrobial compounds in some situations (Verschuere *et al.*, 2000b). Disease problems can be overcome by applying probiotic biotechnology, which is an application of microbial ecology. It makes use of the natural mechanisms by which bacteria compete against each other (Moriarty, 1999).

A probiotic is a single or mixed culture of live, naturally occurring microorganisms that have a positive effect when introduced into tanks, ponds, or administered to the larvae via live food. Primarily, these act by enhancing degradation of organic matter, improving water quality and realising antibacterial and antiviral compounds (Davenport, 2003).

Probiotics have found use in aquaculture as a means of disease control, supplementing or even in some cases replacing the use of antimicrobial compounds. A wide range of microalgae (*Tetraselmis*), yeasts (*Debaryomyces*, *Phaffia* and *Saccharomyces*) and Gram-positive (*Bacillus*, *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Micrococcus*, *Streptococcus* and *Weissella*) and Gram-negative bacteria (*Aeromonas*, *Alteromonas*, *Photorhodobacterium*, *Pseudomonas* and *Vibrio*) has been evaluated (Irianto and Austin, 2002a). According to Verschuere *et al.* (2000b), the addition of probiotics to a culture that is performing under optimal conditions (in the absence of pathogens) may not provide any notable benefit, but the probiotic used as biological control agents will be very helpful if infectious diseases break out. In another study, Gatesoupe (1999) found that sometimes it is possible to have a direct growth promoting effect on fish either by a direct involvement in nutrient uptake, or by providing nutrients or vitamins with probiotics.

The potential probiotics can be administrated during the larviculture to the host, or directly in the environment in different ways (Verschuere *et al.*, 2000b):

- (i) addition to the artificial diet;
- (ii) addition to the culture water;

- (iii) bathing;
- (iv) addition via live food.

2.5.4. Gnotobiotic Systems

Gnotobiotic, derived from the Greek “gnotos” meaning known, and “biota”, together, indicate an animal with associated flora or fauna that is fully defined by accepted current methodology (Wostmann, 1996).

Caesarean section or embryo transfer are necessary to rear axenic animals, defined as animals free of all demonstrable forms of microbial (Yu, 1999). The concept of rearing animals free of microbial associates was first expressed by Louis Pasteur (Tannock, 1995). Axenic organisms cultured in gnotobiotic systems can be used to check the efficacy of new treatments for disease control, to understand the protection mechanisms provided by such treatments and to study the host-microbe and microbe-microbe interactions (Marques, 2005).

2.6. Review about the strains used in the experiments and microbiological constrains

2.6.1. *Escherichia coli*

Escherichia coli belongs to the family Enterobacteriaceae. It is a gram-negative flagellated rod-shaped bacterium. The microorganism is a facultive anaerobe, oxidase negative, indole positive and does not utilize citrate. The microorganism ferments glucose and other carbohydrates, producing acid and gas. Metabolism of glucose results in pyruvate, which is converted to lactic, acetic, and formic acids. Further metabolism of formic acids results in equal amounts of CO₂ and H₂ (Yousef and Carlstrom, 2003).

2.6.2. *Aeromonas hydrophila*

Aeromonas are fermentative, free-living aquatic bacteria that possess a thin capsule and a single polar flagellum. They are present mostly in freshwater interfaces, cold-blooded animals such as fish, snakes, and frogs (for which they may be pathogenic), soil, and certain foods. The *Aeromonas hydrophila* group, consisting of *A. hydrophila*, *A. caviae*, and *A. veronii* biotype *sobria*, are the medically most important members of the genus. Most clinical isolates are susceptible to tetracycline, aminoglycosides, sulfamethoxazole-trimethoprim, and cefamandole. However, they are resistant to penicillin, ampicillin, cephalothin, and other β -lactam antibiotics (Shimeld and Rodgers, 1999).

Motile aeromonads, especially *Aeromonas hydrophila*, can cause septicemia in a wide variety of freshwater fish, but there is some disagreement concerning the role of *A. hydrophila* as a fish pathogen. *Aeromonas hydrophila* is a Gram-negative bacterium, which is motile by a single polar flagellum. It is a rod-shaped organism (0.8-1.0 x 1.0-3.5 μm) which is fermentative and oxidase and catalase positive (Couch and Fournie, 1993).

Gibson *et al.* (1998) has shown that *Aeromonas media* strain A199, an organism isolated from fresh water, is a potential probiotic for use in the aquaculture industry. The results with the application of *Aeromonas media* A199 was found to prevent death of the oyster *Crassostrea gigas* larvae when they were challenged *in vivo* with the pathogen *Vibrio tubiashii*. The addition of *Aeromonas media* A199 to the larvae caused a spectacular decrease of the pathogen densities in the larvae compared to those in the larvae treated with *V. tubiashii* only.

Strains of *Aeromonas hydrophila* isolate from skin lesions of the common freshwater fish, *Tilapia mossambica* tested for susceptibility to 10 antimicrobial agents. Of the 21 fish isolates examined in this study, all were resistant to ampicillin (Son *et al.*, 1997).

The bacterium used in this study was also used by Verschuere *et al.* (2000a). The bacteria strain LVS3 isolated from well-performing *Artemia* cultures, was identified as *Aeromonas hydrophila* by several biochemical tests by Verschuere *et al.* (2000a), and the authors concluded that LVS3 and other strains provide total protection against the pathogenic action of *V. proteolyticus* CW8T2 with an average rate of survival higher than 80%.

Irianto and Austin (2002b) reported that cultures of *A. hydrophila* and *V. fluvialis* were effective in controlling infections by *A. salmonicida* in rainbow trout.

2.6.3. Conjugation

Conjugation allows the unidirectional transfer of genetic material from a donor to a recipient organism via direct cell-cell contact. Conjugative DNA transfer is considered as a major route of genetic exchange in nature, because it permits exchange of genes not only between different bacterial species, but also the transfer of DNA from prokaryotes to eukaryotes, although not the most common (Bitton, 1999; Lengeler *et al.*, 1999).

Cells undergoing conjugation must make direct physical contact, helped by the microscopic tube called the pilus that serves in this transfer of the gene. The overwhelming majority of observed cases of conjugation have involved Gram-negative bacteria. Unlike other mechanisms, conjugation can take place between strains having different physiological characteristics. The self-transmissible small replicons responsible for bacterial conjugation are generally known as conjugation plasmids (Sonea and Panisset, 1983; Lengeler *et al.*, 1999). The large and more important group of conjugative plasmids, the resistance transfer factors, was discovered in the late 1950s and early 1960s in Japan. The R plasmids are responsible for the rapid spread of antibiotic resistance genes among enterobacteria, particularly in hospitals.

2.6.4. Viable but non-culturable (VBNC) state

The viable but non-culturable (VBNC) state is a survival strategy adopted by bacteria when exposed to environmental stress. Some bacteria may respond to adverse conditions by entering a phase whereby they are able to metabolize and survive but they fail to grow on the routine bacteriological media on which they would normally grow and develop into colonies. The hostile environmental conditions that have been described as inducing the activation of the VBNC state include low nutrient concentrations, low or high temperatures, high salinity and extreme pH. The existence of VBNC cells has been described for a wide variety of Gram-negative and -positive bacteria, including vibrios, campylobacters, aeromonads, legionellas and members of the Enterobacteriaceae. In some experiments, it has been demonstrated that these VBNC cells are able to infect animals when introduced experimentally. However, at present, without resorting to *in vivo* experiments, it is impossible to determine if cells that are present in a sample, but unable to grow on culture media are able to reproduce and thus be of concern to human health (Kell and Young, 2000; Fricker, 2002). Studies have now demonstrated conclusively that resuscitation does occur, as demonstrated in *V. vulnificus* (Whitesides and Oliver 1997).

For the same bacteria, studies show a time required for cells to enter the VBNC state of months and other, just days. The “age” of the cells is a factor that has dramatic effect on the time required for lab-grown cells to become non-culturable. Cells from *V. vulnificus* in logarithmic phase of growth generally require less than ten days became completely non-culturable at 5° C, those taken from the stationary phase require over a month (Oliver *et al.*, 1991).

3 MATERIALS AND METHODS

The experiment consists of two parts:

1. Effects of different treatments on viability and bacteria load of the turbot eggs;
2. The effect of different treatments on the survival and on the bacteria load of the larvae obtained in the first part.

3.1. Origin of material:

3.1.1. Turbot eggs

The experiments described below were carried out on artificially stripped and fertilised turbot eggs (*Psetta maxima*) provided by a commercial hatchery (France Turbot, France).

Upon arrival at the culture room (Laboratory of Aquaculture & Artemia Reference Center, Ghent – Belgium), the temperature of the water with the eggs was measured for the acclimatization to the culture water temperature at 15°C. Afterwards, the eggs were transferred into a conical tank for 6 hours, to get the same pH and water composition in 5 L of filtered UV treated seawater. The water was exchanged at a magnitude of 500% using UV treated filtered natural seawater at a salinity of about 35 psu. (practical salinity units). The eggs were lightly aerated to keep them suspended and in a uniform distribution within the water column. Water temperature was maintained at 15°C±1 during the entire experiment.

3.1.2 Preparation of bacterial strains

Pure cultures of *Aeromonas hydrophila* - LVS3 (rifampicin resistant) and *Escherichia coli* - RP4 (ampicillin, tetracycline and kanamycin resistant) strains were provided by the Laboratory of Microbial Ecology and Technology, Ghent University. The bacteria were plated on Luria-Bertani (LB) agar, each on your respectively plate, and incubated for 24 hours at 28°C. The grown colonies were transferred to 20 ml of

autoclaved LB broth for respectively bacteria strain and incubated for 24h at 28°C in a gyratory shaker (Model G76, New Brunswick Scientific, USA) at 200 rpm. From each bacteria liquid culture, serial dilutions of 10^{-4} , 10^{-5} , 10^{-6} and 10^{-7} were prepared in sterile (autoclaved at 120°C for 20 min.) physiologic solution (0.85% NaCl). All dilutions from both strains were plated (50µl volume in each plate) by a spiral plater (Spiral Systems Inc.) on LB agar with: kanamycin (50 mg/l); ampicillin (50 mg/l), tetracycline (20 mg/l); and rifampicin (50 mg/l). All plates were incubated for 24h at 28°C and 42°C to check the antibiotic resistance of LVS3 and RP4.

For the bacteria conjugation a mixture of 50 ml from LVS3 and RP4 LB liquid culture was made and centrifuged (Sanyo Harrier 18/80) at 10000 rpm for 10 minutes, the resulting pellet was resuspended with a flat-bed vortexer (VWR International, USA) in 50µl of physiologic solution. The 50 µl was spotted on LB agar and incubated at 28°C overnight. The colonies on the LB agar plate were transferred to different media with or without antibiotics to test the conjugation efficiency rate: LB agar; LB agar with rifampicin (50 mg/l, Sigma); LB agar with rifampicin (50 mg/l) and ampicillin (50 mg/l, Sigma); LB agar with rifampicin (50 mg/l) and tetracycline (20 mg/l, Sigma); LB agar with rifampicin (50 mg/l) and kanamycin (50 mg/l, Sigma); Mac Conkey agar (Biokar diagnostics); and Mac Conkey with ampicillin (50 mg/l).

From the plate with rifampicin and tetracycline, colonies were taken and transferred to an erlenmeyer with 50 ml of LB broth and incubated overnight at 28°C on an orbital shaker. From this liquid culture we transferred 20ml divided into two falcon tubes. The liquid was centrifuged three times (5000 rpm for 5 minutes) and the pellet resuspended with 5 ml sterile Nine Salt Solution (NSS; Olsson *et al.*, 1992), every time. An aliquot of 100µl was taken and suspended in 900µl of autoclaved NSS from this liquid culture. The density of bacterial suspension was determined by use of a spectrophotometer (Genesys TM 20, Thermo Spectronic, USA) and a reading was done at a wavelength of 550 nm. NSS was used as blanc and the optical density measured in the range of 0,1 to 0,4. The formula used to determine the bacterial density was:

Optical density (measured) x 1200 x 10^6 x dilution factor = million of bacteria/ml

3.2 Experiments on the effect of different chemical treatments on the viability and bacteria load of turbot eggs

Two experiments were performed at a different date, different stocking procedures and addition of different antibiotics; they are cited as first experiment and second experiment, respectively.

3.2.1 Initial procedures

At the end of the acclimatization of the eggs and rising with UV treated seawater, a batch of 12.000 eggs was collected on a nylon sieve of 300 μm mesh size and washed 4 times with 1 litre autoclaved seawater to remove organic materials. The eggs density was measured by taking 6 samples of 10 ml from the large conical tank. All procedures with turbot eggs and larvae were conducted in the culture room at $15^{\circ}\text{C}\pm 1$ with a thermostat to keep the temperature constant.

3.2.1.1 First experiment on the effect of different chemical treatments on the viability and bacteria load of turbot eggs

The eggs were stocked in 4 conical flasks for each treatment (fig. 3), each conical flask containing 800 ml of autoclaved (120°C for 20 min) seawater at a density of 750 eggs/liter. The set up consisted of 12 conical flasks stored in the culture room with controlled temperature at $15^{\circ}\text{C}\pm 1$.

Firstly, the eggs for the control treatment were stocked, and the rest was disinfected. The procedures in all experiments were conducted inside the laminar air flow chamber to avoid contamination. The egg experiment was performed with continuous light and aeration. The conical flasks were autoclaved with aluminium foil to cover the opening. Aeration was provided through sterile pipes fitted with filters (membrane with 0,2 μm mesh size) to avoid external contamination.

Upon arrival, 2 samples of 30 eggs each were taken using a sterile 25 ml pipette after rinsing and more 2 samples of 30 eggs after disinfection. The samples were taken in replicates and kept in sterile sampling bottles for microbial analysis.

3.2.1.2 Egg disinfection

For the disinfection, the eggs were submerged using a nylon sieve of 300 μm mesh size in 500 ml autoclaved seawater with 200 mg/l of glutaraldehyde [50% (v/v) in water (Fluka)], in water using a 1 liter beaker for 5 minutes.

3.2.1.2.1 Disinfection efficiency test

The eggs were transferred aseptically to a sterile plastic bag, resuspended in 10 ml sterile NSS and homogenized in a Stomacher Lab. Blender (Seaward, London, UK) for 120 seconds at normal speed and 120 seconds at high speed. From the homogenized samples, serial dilutions of (10^0 , 10^{-1} , 10^{-2} and 10^{-3}) were prepared in sterile NSS and plated on Marine agar (MA, Difco). All plates were incubated at 28°C and the colony forming units (CFU) counted after 24, 72 and 120 hours, respectively. The number of colonies counted was expressed as CFU/egg. On the second day, the same experiment was conducted for microbial analysis using 3 x 30 eggs from each treatment in duplicates.

According to Gunasekaran (1995), the standard method for count plate should contain between 30 and 300 colonies for accuracy in counting. In the results, the plates with more than 300 CFU were expressed as >2000 CFU/egg and for less than 30, <200 CFU/egg respectively.

3.2.1.3 Egg disinfection followed by addition of antibiotic

For the disinfection with antibiotics treatments, we use a part of the eggs that was disinfected with glutaraldehyde. For this treatment were added 10 mg/l of tetracycline, ampicillin, rifampicin and kanamycin (Fig. 3).

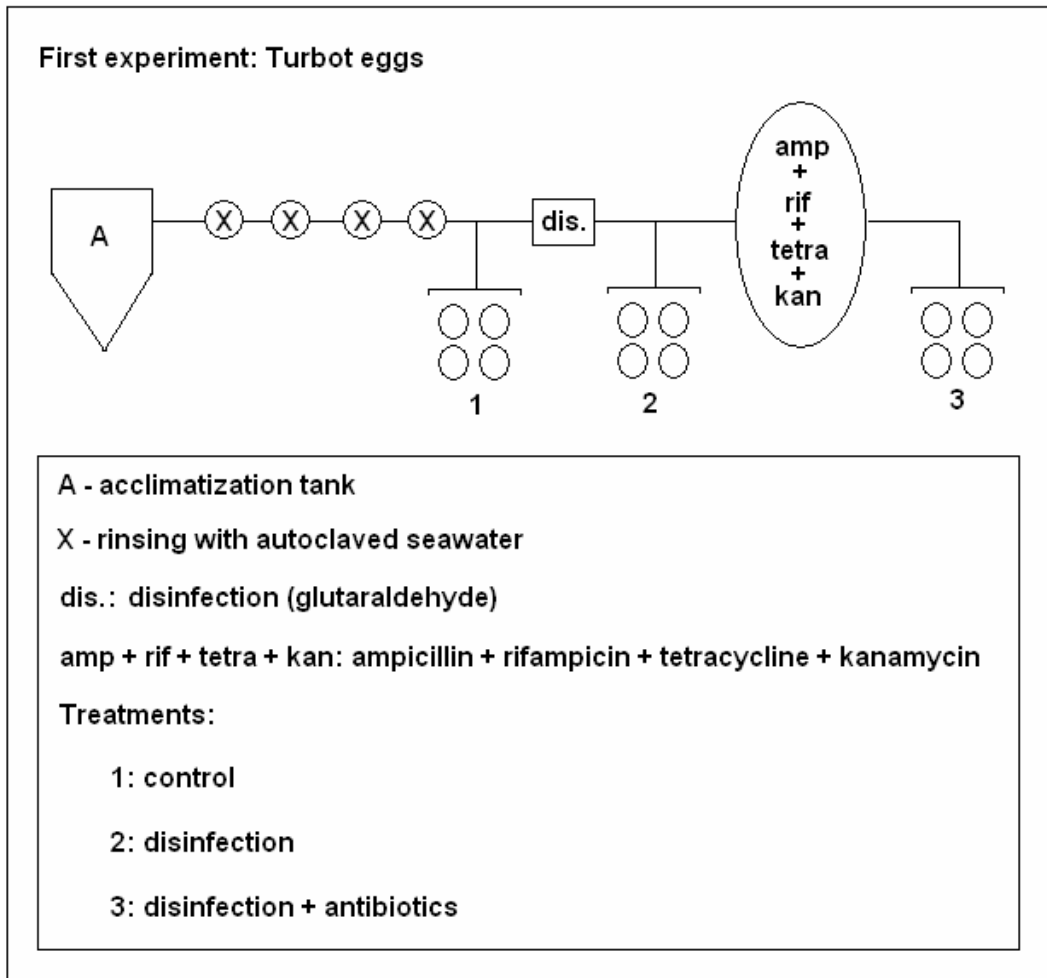


Fig. 3 Steps conducted with the turbot eggs in the first experiment with the different treatments.

3.2.1.4 Hatchability efficiency test

The hatchability of the eggs was determined taking 3 samples of 5ml (with a 25ml pipette) from each conical flask in all treatments after 24 hours since the first eggs hatched in the experiment. The rate of hatched eggs is given as larvae/ml.

3.2.2.1 Second experiment on the effect of different chemical treatments on the viability and bacteria load of turbot eggs

The eggs were stocked in 4 small glass bottles for each treatment, each glass bottle containing 400 ml of autoclaved seawater at a density of 750 eggs/liter.

Firstly, the eggs for the control treatment were stocked, and the rest was disinfected.

The procedures in all experiments were conducted inside of the laminar flow to avoid contamination. The egg experiment was performed with continuous light and aeration. The set up consisted of 32 sterilized glass bottles (Fig. 4) stored in the culture room with controlled temperature at $15^{\circ}\text{C}\pm 1$. The glass bottles were autoclaved with the respectively caps and pipes. Aeration was provided through pipes fitted with filters (membrane with $0,2\ \mu\text{m}$ mesh size) to avoid external contamination.

Upon arrival, 2 samples of 30 eggs each were taken using a sterile 25ml pipette after rinsing and more 2 samples of 30 eggs after disinfection. The samples were taken in replicates and kept in sterile sampling bottles for microbial analysis.



Fig. 4 Experimental set-up showing the bottles, filters and aeration system

3.2.2.2 Egg disinfection

For the disinfection, the eggs were submerged using a nylon sieve of $300\ \mu\text{m}$ mesh size in 500 ml autoclaved seawater with 200 mg/l [50% (v/v) in water] of glutaraldehyde using a 1 liter beaker for 5 minutes.

3.2.2.2.1 Disinfection efficiency test

The eggs were transferred aseptically to a sterile plastic bag, resuspended in 10 ml sterile NSS and homogenized in a Stomacher Lab. Blender (Seaward, London, UK) for 120 seconds at normal speed and 120 seconds at high speed. From the homogenized samples, serial dilutions of (10^0 , 10^{-2} , 10^{-4} and 10^{-6}) were prepared in sterile NSS and plated on MA. All plates were incubated at 28°C and the colony forming units (CFU) counted after 24, 72 and 120 hours, respectively. The number of colonies counted was expressed as CFU/egg. On the second day, the same experiment was conducted for microbial analysis using 3 x 30 eggs from each treatment in replicates.

3.2.2.3 Egg disinfection followed by addition of antibiotic

For the disinfection with antibiotics treatments, we use a part of the eggs that was disinfected with glutaraldehyde. For each subsequent treatment, 10 mg/L of each antibiotic was added. For this experiment we used ampicillin in the treatment number 3 and 6, rifampicin in the number 4 and 7, and rifampicin in addition with ampicillin in the number 5 and 8, as shown in the figure 5.

3.2.2.4 Experiment on the effect of addition of LVS3-RP4 transconjugant on the viability and bacteria load of turbot eggs within different treatments

This experiment is the continuation of the second experiment cited above. The addition of LVS3-RP4 transconjugant in the bottles 6, 7 and 8 (Fig 5) previously treated with disinfection and antibiotic/antibiotics was conducted on the first experiment day and on the hatching day, at a concentration of 10^5 CFU/ml.

At the first day of the egg experiment, 10^5 CFU/ml LVS3-RP4 transconjugant was added in each bottle from the treatments 6, 7 and 8 and after hatching, 10^5 CFU/ml of LVS3-RP4 was added again.

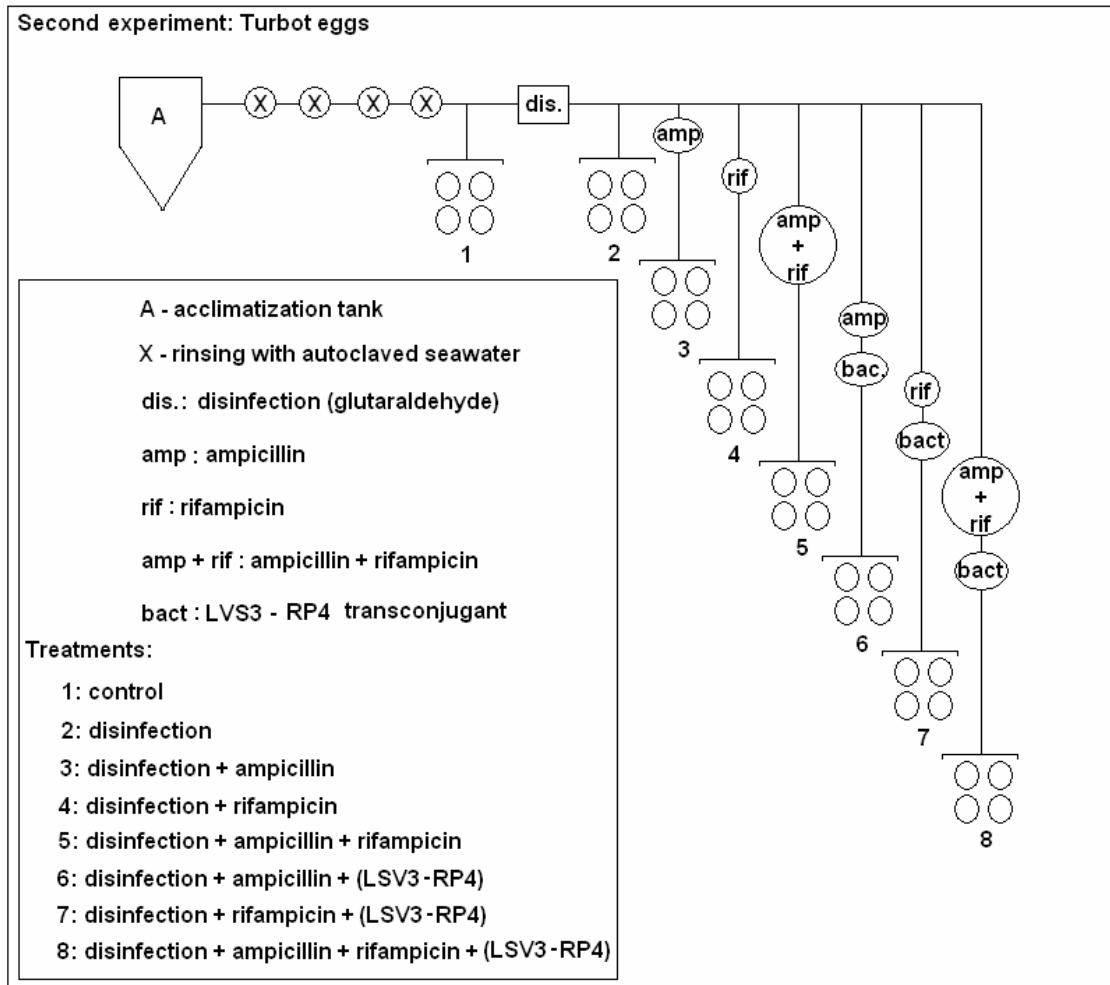


Fig. 5 Steps conducted with the turbot eggs in the second experiment with the respective treatments

3.2.2.5 Hatchability efficiency test

The hatchability of the eggs was determined taking 3 samples of 5ml (with a 25ml pipette) from each glass bottle in all treatments after 24 hours since the first eggs hatched in the experiment. The rate of hatched eggs is given as larvae/ml.

3.3 Larvae survival experiment

The larvae survival experiment starts after hatching. The larvae were kept without food to conserve the axenic environment. No aeration was provided in the larvae survival experiment.

Larvae from each hatching cone (first experiment) or glass bottle (second experiment), were transferred into the respective beakers. Each beaker was filled with 500 ml of autoclaved seawater.

The beakers were autoclaved with an aluminium foil to cover the opening. The aluminium foil serves like a cap for the beaker. The beakers were placed inside a non-transparent PVC pipe to reduce the light intensity during all the survival experiment. The temperature during all the experiment was the same, 15°C±1.

3.3.1 Larvae survival experiment 1

In the first experiment, the larvae were transferred to beakers with autoclaved seawater for all treatments (fig. 6).

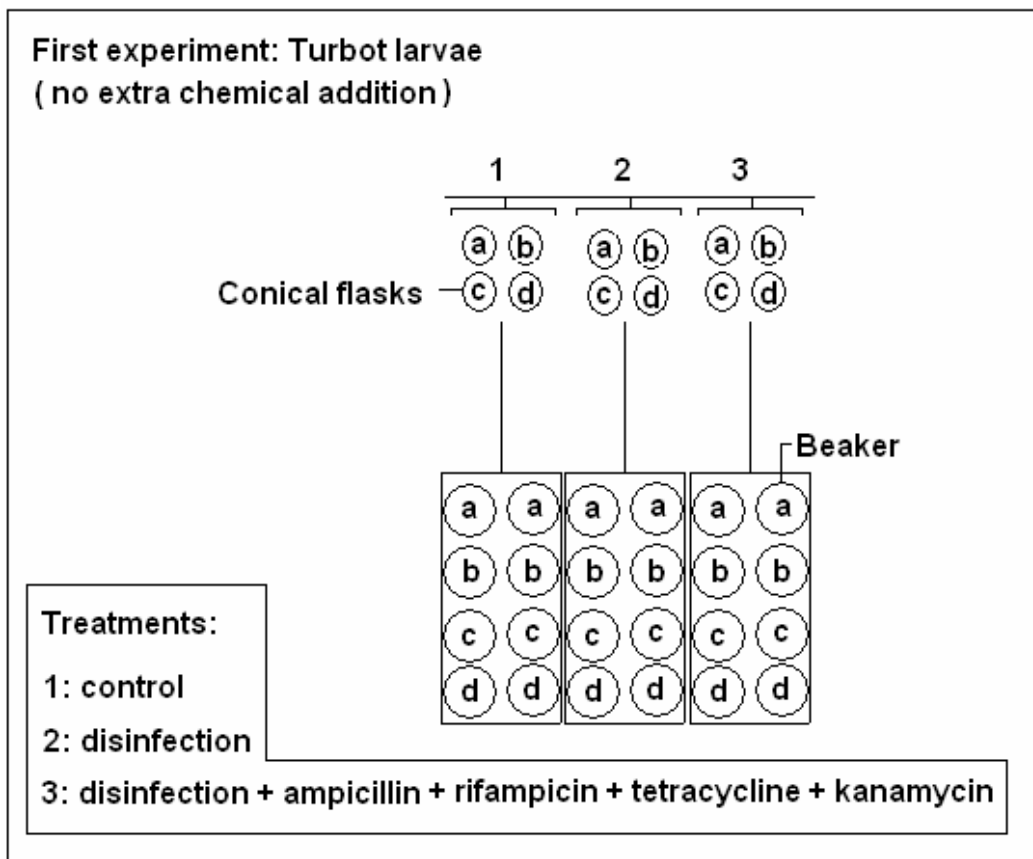


Fig. 6 Steps conducted with the turbot larvae in the first experiment with the different treatments

The larvae were counted daily for 4 days to determine cumulative mortality in each beaker in all treatments. On the fourth day, the larvae were rinsed 4 times in

beakers with PBS (using a sieve 300 µm mesh size) for the histological analyses. The larvae were centrifuged for 1 minute at 1000 rpm and resuspended in PBS 3 times. The last time the PBS was exchanged for fixation liquid in 10x the previous volume. The histological analyses were conducted at Faculty of Veterinary immediately after fixation.

3.3.2 Larvae survival experiment 2

In the second experiment, the larvae were transferred from each hatching glass bottle into the respective beakers.

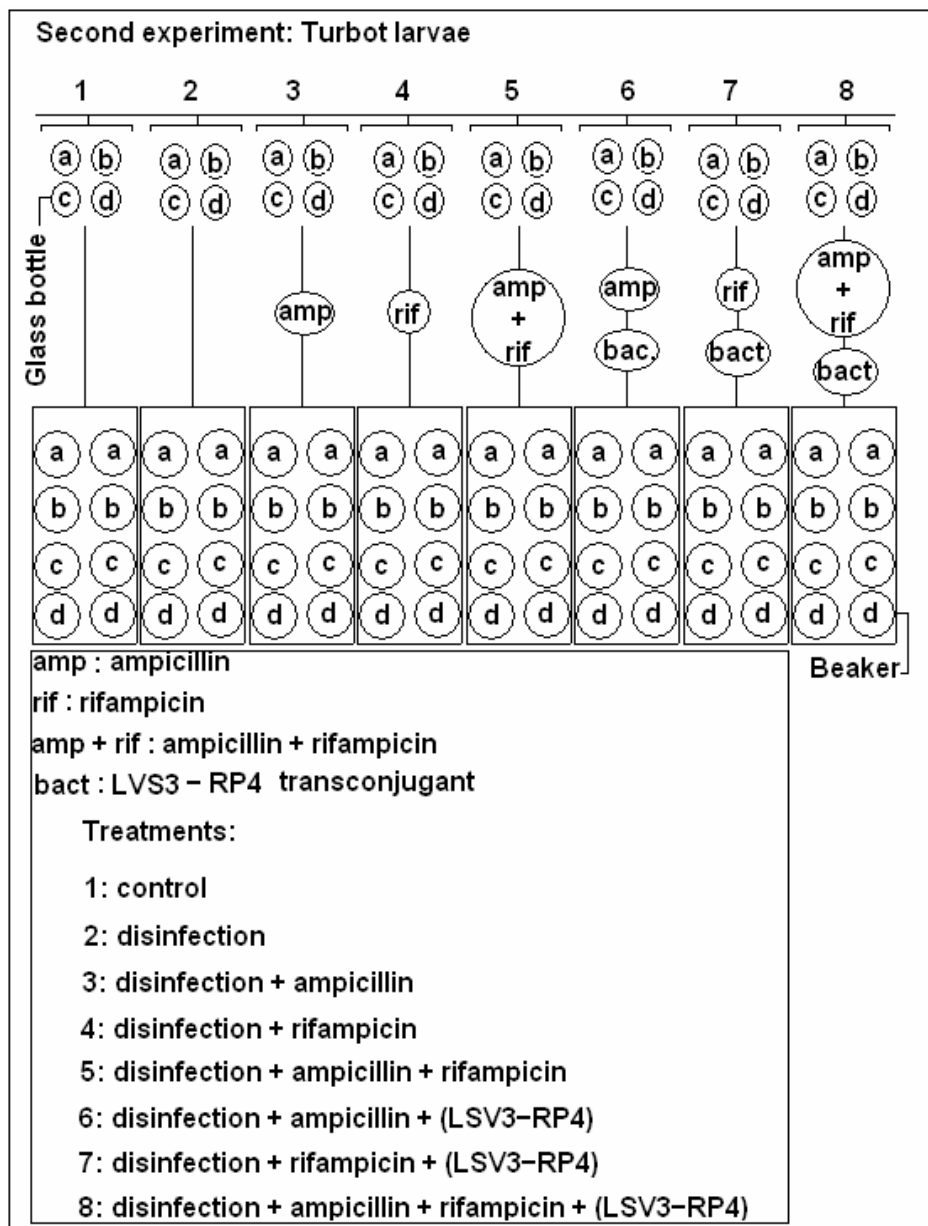


Fig. 7 Steps conducted with the turbot larvae in the second experiment with the different treatments

The beakers were previously filled with 500 ml of autoclaved seawater with or without antibiotic/antibiotics and with or without LVS3-RP4 transconjugant as respectively previous eggs experiment. The concentrations of antibiotics were the same: 10 mg/L of ampicillin was added again in the treatment 3 and 6; in the treatment 4 and 7, 10 mg/L of rifampicin, and in the treatment 5 and 8 were added 10 mg/L of ampicillin and 10 mg/L of rifampicin as shown in the figure 7. In all beakers 10^5 CFU/ml of LVS3-RP4 transconjugant were added on the treatment 6, 7 and 8 (Fig 7).

The larvae were counted daily for 3 days to determine cumulative mortality in each beaker in all treatments. On the third day, the larvae were rinsed 4 times in beakers with PBS (using a sieve 300 μ m mesh size) for the histological analyses. The larvae were centrifuged for 1 minute at 1000 rpm and resuspended in PBS 3 times. The last time the PBS was exchanged for fixation liquid in 10x the previous volume. The histological analyses were conducted at Faculty of Veterinary immediately after fixation.

3.4 Bacterial development experiment

Colonies of LVS3-RP4 transconjugant were plated on MA at 28°C and 16°C. The grown colonies, after 7 days, were washed with 20 ml of filtered and autoclaved seawater into erlenmeyers for each respective temperature.

The seawater used to wash the plate at 28°C was centrifuged at 28°C at 5000 rpm for 10 minutes such as the plate at 16°C (centrifuged at 16°C). The pellet was washed once with filtered and autoclaved seawater for each liquid culture. With a vortex, the pellet was resuspended. The density of bacterial suspension was determined by use of a spectrophotometer and a reading was done at wavelength of 550 nm. Filtered and autoclaved seawater was used as blanc and the optical density measured in the range of 0,1 to 0,4.

Erlenmeyers with 25ml of filtered and autoclaved seawater, 10^5 CFU/ml of LVS3-RP4 transconjugant were added from the resting cells at 16°C. Erlenmeyers with 25ml of filtered and autoclaved seawater, 10^5 CFU/ml of LVS3-RP4 transconjugant

were added from the resting cells at 28°C. The erlenmeyers stayed on a shaker during all experiment at respective temperature. Both treatments were done in replicates.

3.4.1 Experiment on colony formation rate (hours)

In one experiment, 50µl from each erlenmeyer stored at 28°C, in replicates, were plated on MA with a spiral plater and incubated at 16°C and 28°C after 30 minutes, 1 hour, 2 hours, 4 hours and 8 hours respectively. The number of CFU counted after 24, 48, 72 and 96h.

3.4.2 Experiment on colony formation rate (days)

The second experiment was done with the same seawater with LVS3-RP4, but the samples were plated in MA every day. From the erlenmeyers with seawater at 28°C were plated with a spiral plater and incubated at 28°C. From erlenmeyers with seawater at 16°C were plated with spiral plater and incubated at 16°C and 28°C. The number of CFU counted after 24, 48, 72 and 96h for the plates incubated on 28°C and for the plates incubated at 16°C the counting were conducted for 15 days (360h).

3.4.3 LVS3-RP4 transconjugant counts experiment between the different antibiotics

In this experiment, were used autoclaved small glass bottle with respective cap with 400 ml of autoclaved seawater, for each treatment.

Colonies of LVS3-RP4 transconjugant were taken and transferred to an erlenmeyer with 50ml of LB broth and incubated overnight at 28°C on an orbital shaker. From this liquid culture we transferred 20ml divided into two falcon tubes. The liquid was centrifuged three times (5000 rpm for 5 minutes) and the pellet resuspended with 5 ml sterile NSS every time. An aliquot of 100µl was taken and suspended in 900µl of autoclaved NSS from this liquid culture. The density of bacterial suspension was determined by use of a spectrophotometer and a reading was done at a wavelength of 550 nm. NSS was used as blanc and the optical density measured in the range of 0,1 to

0,4. In each bottle were added, LVS3-RP4 transconjugant at a concentration of 10^5 CFU/ml. The treatments were: rifampicin with ampicillin; rifampicin; ampicillin. The antibiotic concentration was the same use in the turbot experiment, 10 mg/l for each antibiotic.

The resting cells into NSS, were kept at 16°C or 28°C for one or two days, and plated on MA or MA 10% with a spiral plater. The incubation temperature for the plates was 28°C and the CFU were counted after 24 and 48h.

3.4.4 LVS3-RP4 transconjugant qualitative experiment on different media

One experiment was conducted using seawater and turbot larva from the turbot experiment glass bottles. The qualitative experiment was done with different media: MA, MA 1%, MA 10%, MA with rifampicin and ampicillin (50mg/l of each antibiotic) and Fish's Agar (normal agar with the addition of turbot larvae, triturate and filtered by mesh size of 200 μ m).

In each plate was homogenizes one larvae and one drop of seawater from respective treatment on turbot experiment. The results are expressed for positive or negative CFU on respective plate. The plates were incubated for 48h at 28°C.

3.5 Statistical analysis

A median test or Kruskal Wallis Test were used for hatch data and for survival data were used Kruskal Wallis test at a significant level of $p < 0,05$. The data were analysed using SPSS software version 12.0 (2003) for windows.

4 RESULTS AND DISCUSSION

4.1 Conjugation efficiency

The antibiotic resistance was checked before the conjugation experiment, and *A. hydrophila* LVS3 was found to be resistant to rifampicin and ampicillin, while the *E. coli* RP4 showed resistance to tetracycline, kanamycin and ampicillin (Fig. 8).



Fig. 8 Colonies of *E. coli* RP4 on Mac Conkey agar with ampicillin (50 mg/l)

The results of the conjugation experiment (Fig.9) represent the efficiency of conjugation between the *Aeromonas hydrophila* (LVS3) and *Escherichia coli* RP4. This conjugation provides a LVS3-RP4 transconjugant resistant to tetracycline, kanamycin, ampicillin and rifampicin.

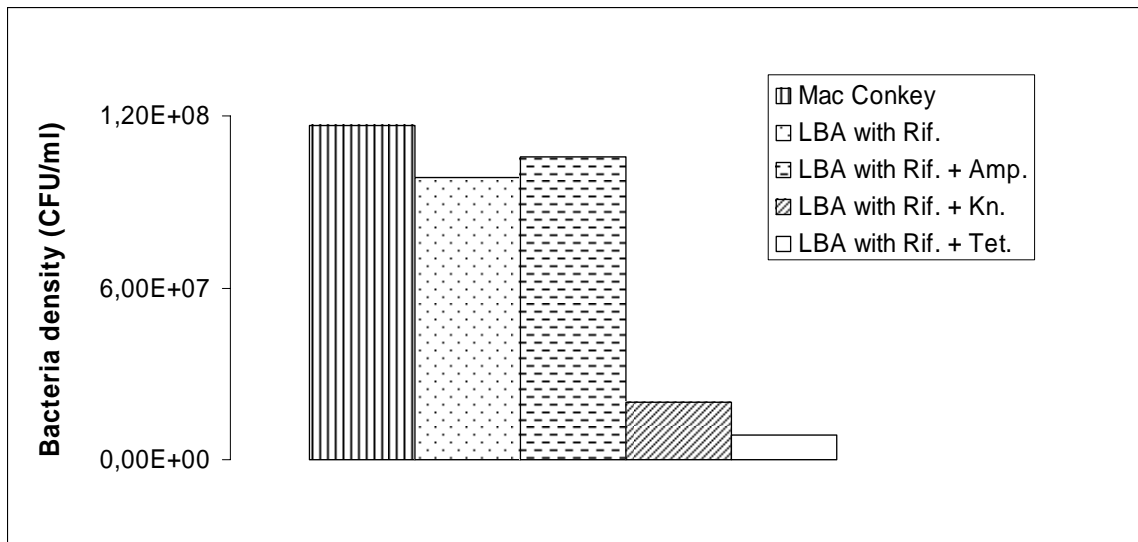


Fig. 9 Conjugation between *Aeromonas hydrophila* (LVS3) and *Escherichia coli* (RP4): CFU/ml on different culture media

On the Mac Conkey media, only the *E. coli* RP4, the donor of the RP4 plasmid was able to grow. On LBA media with rifampicin and LBA with rifampicin and ampicillin, *Aeromonas hydrophila* (LVS3) and the transconjugant (LVS3-RP4) were enumerated. Donor and acceptor were present in more or less equal amount of cells in the conjugation mixture. As LVS3 and LVS3-RP4 are both ampicillin resistant it is not possible to distinguish between acceptor (LVS3) and transconjugant (LVS3-RP4). On the media LBA with addition of rifampicin and kanamycin and LBA with rifampicin and tetracycline, only transconjugants were able to grow. Hence the results of this plating allow to calculate the conjugation efficiency which was 6,58%.

4.2 Disinfection efficiency

4.2.1 First experiment

On the first day, 2 samples of 30 rinsed eggs and more 2 samples of 30 rinsed and disinfected eggs were taken for measuring the disinfection efficiency. The bacterial counts after rinsing were, for all the dilutions, more than 300 colony forming units (CFU) per egg (Table 1). Plates with more than 300 CFU were expressed as >2000 CFU/egg and for less than 30, <200 CFU/egg respectively.

Table 1. Mean bacterial counts (CFU/egg) after rinsing and disinfection, on MA agar at start of experiment 1

Incubation time of MA plates	24h	72h	120h
After rinsing	>2000	>2000	>2000
After disinfection	>2000	>2000	>2000

According to Jones (1972), live eggs of turbot are transparent and buoyant, while dead eggs become opaque and sink. The quality of the eggs used on the first experiment was not like the description cited above for a good egg. Some eggs were already dead before arrival, and the eggs' floatability seemed not so good as well. This fact could be the cause of the huge bacteria load on turbot eggs in this experiment (Table 1).

Even after disinfection, the bacteria load was still very high, and this result probably was caused by the bad egg quality.

On the second day, the bacterial load of the eggs was checked for each treatment in 2 replicates. Between the different chemical treatments, antibiotics showed a better antimicrobial efficiency. The control and disinfection treatment showed, like the day before, a high bacteria load (Table 2).

Normally, the glutaraldehyde should be sufficient for surface disinfection of turbot eggs according to Salvesen and Vadstein (1995), but in our experiment, the disinfection treatment does not indicate any reduction compared to the control (Table 2). Further dilution of the samples (which was not done as better disinfection efficiency was anticipated) could have given a better indication of the disinfection efficiency of the various treatments.

The antibiotic treatment, demonstrates a high disinfection of egg surface and the result counts (<200 CFU/egg) probably were contamination (Table 2).

Table 2. Mean bacterial counts (CFU/egg) for the control, disinfection and antibiotics treatment on the second day, on MA at start of experiment 1. “Incubation time” refers to the time the plates were incubated at 28°C

Incubation time of MA plates	24h	72h	120h
Control	>2000	>2000	>2000
Disinfection	>2000	>2000	>2000
Antibiotics	<200	<200	<200

4.2.2 Second experiment

Upon arrival, 2 samples of 30 rinsed eggs and more 2 samples of 30 rinsed and disinfected eggs were taken for testing the disinfection efficiency. After rinsing, the bacteria counts were very low for the first dilution and zero for others dilutions (10^{-2} , 10^{-4} and 10^{-6}). Plates with more than 300 CFU were expressed as >2000 CFU/egg and for less than 30, <200 CFU/egg respectively.

The difference between the results of experiment 1 and experiment 2 was very high. This reflects a relation between the egg quality and the bacteria load. The bacteria encountered on experiment 2 were contamination (Table 3).

Table 3. Mean bacterial counts (CFU/egg) after rinsing and disinfection, on MA agar at start of experiment 2. “Incubation time” refers to the time the plates were incubated at 28°C

Incubation time of MA plates	24h	72h	120h
After rinsing	<200	<200	<200
After disinfection	<200	<200	<200

On the second day, the bacterial load of the eggs was checked for each treatment in 2 replicates. The number of CFU/ml on the control treatment had increased in comparison to day one, evidencing bacteria development (Table 4). For the disinfection treatment with glutaraldehyde, the counts were very low after disinfection on day one and on day two.

Glutaraldehyde like other compounds in the aldehyde family possesses carbonyl groups that react readily with nucleic acids and proteins (Rutala, 1990). Glutaraldehyde can cross-link amine groups on the outer cell walls and cell membranes of microorganisms, and is believed to be responsible for its efficacy against Gram-negative bacteria, fungi, and viruses (Chambon et al., 1992).

The other disinfection treatments were very efficient. No bacteria were found on eggs disinfected with glutaraldehyde and subsequently treated with ampicillin, rifampicin or a combination of rifampicin and ampicillin.

Table 4. Mean bacterial counts (CFU/egg) for the different treatments on the second day, on MA agar at start of experiment 2 (control mean bacterial counts was between 200 and 2000 CFU/egg \pm standard deviation)

Incubation time of MA plates	24h	72h	120h
Control	1240 \pm 504	1289 \pm 504	1329 \pm 504
Disinfection	<200	<200	<200
Ampicillin	0	0	0
Rifampicin	0	0	0
Rifampicin with ampicillin	0	0	0
Rif. with LVS3-RP4	0	0	0
Amp. with LVS3-RP4	<200	<200	<200
Rif. with amp. With LVS3-RP4	<200	<200	<200

The results for bacterial load in rifampicin with LVS3-RP4, ampicillin with LVS3-RP4 and rifampicin together with ampicillin and LVS3-RP4 treatments have shown an unexpected number of colonies/egg.

In the experiment, 10^5 CFU/ml were added on the first day and one day later. No bacteria were found back in the “rifampicin with LVS3-RP4” treatment, and a very low number in the “ampicillin with LVS3-RP4” treatment, and in “rifampicin with ampicillin with LVS3-RP4” treatment (Table 4). This could be explained by the inability of LVS3-RP4 to attach to the eggs.

The viability of a bacteria cell was traditionally determined by its ability to produce colonies. However, many studies have revealed the ability of both Gram-positive and Gram-negative bacteria, to go into a viable but non-culturable (VBNC) state. In this state, bacteria are still viable and show metabolic activity and respiration, but cannot be enumerated as colony forming units by the conventional plate counts and hence remain hidden (Colwell and Grimes., 2000). Environmental stress can induce this VBNC state. In this study the environmental stress could have been caused by a rapid decrease in incubation temperature. The LVS3-RP4 cells were grown at 28°C in Marine Broth and subsequently transferred to 16°C for incubation together with the turbot eggs. So it is conceivable that the LVS3-RP4 transconjugant was entering in VBNC state because of the stress caused by the rapid temperature decrease during the handling. In addition, although hypothetically, the ability of the bacteria to attach to the egg surface could have been affected resulting in an apparent low or zero LVS3-RP4 transconjugant load (Table 4).

4.3 Hatchability results

4.3.1 First experiment

In the hatchability efficiency test no significant differences were observed ($p > 0,05$; Median test) between the control and the disinfection treatments. There was a significant difference between disinfection and disinfection with antibiotics ($p < 0,05$; Median Test), as well as between the control and the disinfection with antibiotics. The disinfection with antibiotics treatment was significantly different from the other treatments.

The disinfection with antibiotics treatment consisted in the addition of 4 different antibiotics: ampicillin, rifampicin, kanamycin and tetracycline. The teratogenic effect of tetracycline is possible for mammals according to Gomella *et al.*, (1999). Oxytetracycline is the most commonly used member of the tetracycline group and is probably the most widely used antibiotic in aquaculture. They are inexpensive antibiotics, which have been used extensively in the prophylaxis and therapy of human and animal infections, and also at subtherapeutic levels in animal feed as growth

promoters. In aquaculture, it can be applied orally or as a bath, and is effective against a wide range of Gram-negative and Gram-positive bacteria such as *Aeromonas* or *Vibrio* sp. in fish and crustaceans (Capone *et al.*, 1996; Smith, 1996). The possible teratogenic effect of kanamycin is registered in literature, but less evidenced than the teratogenic effect of tetracycline (Fabre *et al.*, 2002). No bibliographies were found for the possible teratogenic effect on hatchability or fish larvae development after application of tetracycline or kanamycin on eggs.

According to the results found by Kjørsvik *et al.* (2003), initial egg quality is crucial for the quantitative and qualitative juvenile production of turbot, and egg quality may possibly affect the juvenile production of other marine species as well. Variations in egg quality may thus explain some of the differences which are often observed between productions of larval groups reared under similar conditions. The overall big difference in egg hatching between experiment 1 (Fig. 10) and experiment 2 (Fig. 11) is most probably a reflection of the difference in quality.

Ecotoxicity tests on embryos of *Oncorhynchus mykiss* demonstrated sensitivity with daily exposures to glutaraldehyde at concentration of 2.5 mg/l resulting in a 97% reduction in hatch rate. Even at concentrations as low as 1.3 mg/l, embryos had difficulty emerging from the chorion (Sano *et al.*, 2005). The eggs from plaice (*Pleuronectes platessa* L.) were more resistant to bursting in tests of sublethal damage when treated with glutaraldehyde at a concentration of 1600 mg/l (25% solution) in comparison to lower concentration treatments (Salvesen and Vadstein, 1995). The results showed an embryo unable to leave the chorion, which may be due to increased hardening of the egg chorion, as the principal reason for the failure of hatching.

According to Sano *et al.* (2004), there is strong evidence that glutaraldehyde toxicity will be temperature dependent. Lower environmental temperatures partially mitigate toxicity while higher temperature increases it (Landrum *et al.*, 2003).

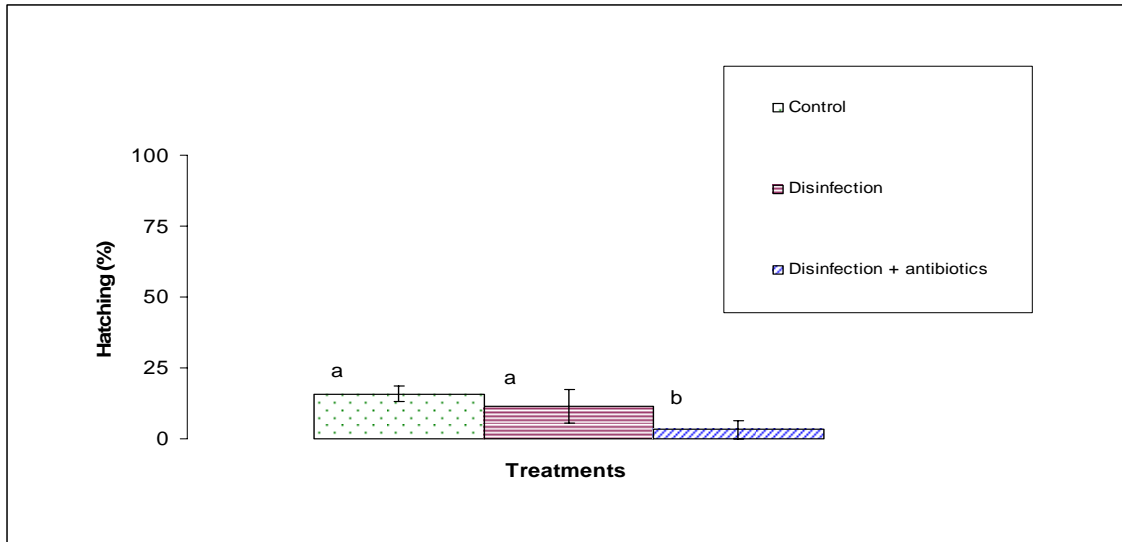


Fig. 10 Mean hatching percentage \pm standard deviation of the eggs of experiment 1. For each treatment, 4 replicas were conducted. Equal letters indicate no significant different result ($p > 0,05$; Median Test)

4.3.2 Second experiment

The results of the hatchability efficiency test were analysed with the Kruskal Wallis Test. The percentage hatching is higher than 100% because of an underestimation of the egg density on the arrival day (Fig. 11). However relative differences stay detectable.

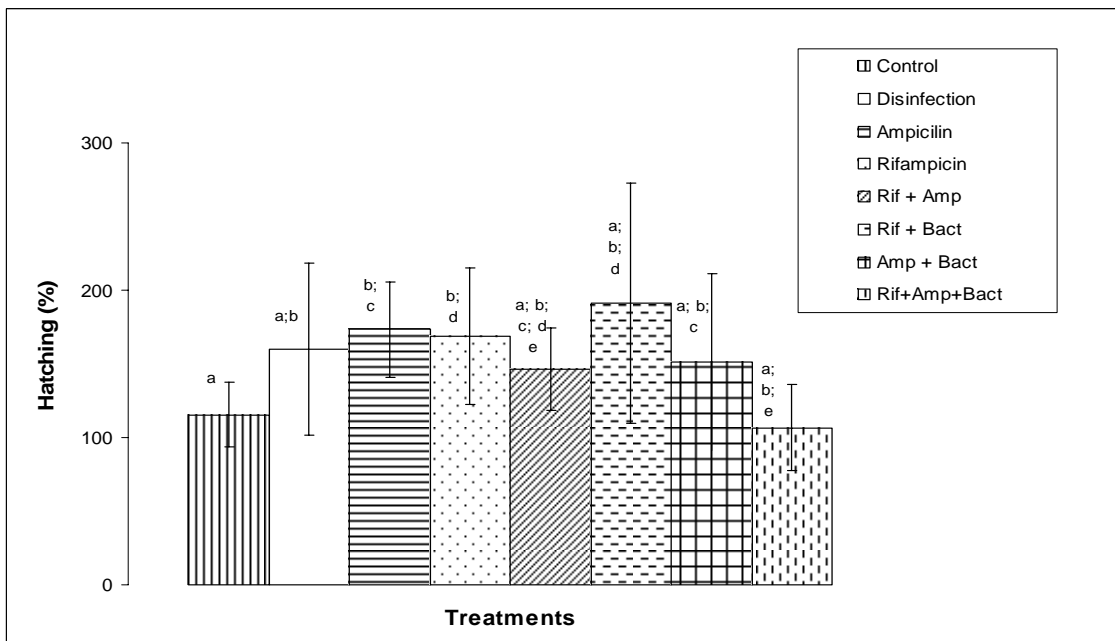


Fig. 11 Mean hatching percentage \pm standard deviation. For each treatment, 4 replicas were conducted. Equal letters indicate no significant difference between the treatments ($p > 0,05$; Kruskal Wallis Test)

Eggs of spotted wolffish (*Anarhichas minor*) were disinfected with glutaric dialdehyde (150 mg/l for 5 min) once or twice a month and showed a prematurely hatched larvae when eggs were disinfected twice a month compared to once a month at all incubation temperature regimes (Hansen and Falk-Petersen, 2001).

In this study, the hatching had occurred within approximately 24h for all treatments.

In this experiment, the hatching percentage was higher (Fig. 11) compared with the first hatching experiment (Fig. 10). These findings demonstrated that indeed, egg quality showed a strong relation with hatchability.

The hatching (Fig. 11) percentage showed no significant difference between the control, disinfection, “ampicillin with bacteria” and “rifampicin with ampicillin with the addition of LVS3-RP4 treatments”. All the treatments, with exception of the control, were previously treated with glutaraldehyde. No significant differences were found between the treatments “rifampicin”, “rifampicin with ampicillin”, “rifampicin with LVS3-RP4” and the same result were obtained for “ampicillin”, “ampicillin with rifampicin” and “ampicillin with LVS3-RP4”.

For coldwater fish eggs, Salvesen and Vadstein (1995) have found a safe and effective level for surface disinfection using glutaraldehyde at a concentration of 400 mg/l (25% solution) for 10 minutes increasing the percentage of hatch and survival in comparison with the control.

According to Peck *et al.* (2004), the use of commonly available disinfectants can improve the hatchability of Atlantic cod and haddock eggs. In combination with an antibiotic solution (streptomycin/penicillin) or with PVP iodine the results were even better.

No differences were found between the treatment with the addition of LVS3-RP4 and the same treatments without the bacterium. This could be another evidence for the entrance of this bacterium in VBNC state. Alternatively LVS3-RP4 might not have any effect at all on egg hatching.

4.4 Survival results

4.4.1 First experiment

The results for the larval survival in the first experiment is given in Fig. 12. The statistical analyse indicated no significant differences ($p>0,05$, Kruskal Wallis Test) on the day 1. On the second, third and fourth day, the disinfection treatment was significantly different from the other treatments ($p<0,05$).

The disinfection treatment showed a better result in larval survival (Fig. 12). Salvesen and Vadstein (1995) have also found a significant increase in survival, by treating Atlantic halibut (*Hippoglossus hippoglossus* L.) eggs with glutaraldehyde.

There was no teratogenic effect on the larval survival in this study. According to Cooper and McGeorge (1991), it is known that some chemicals are more toxic to eggs than to juvenile fish, and vice versa.

The egg quality may interfere with the larval survival in this experiment. The high bacterial load could mark possible differences between the treatments. The big load of bacteria on the egg surface may have lead to a decrease of survival in the control treatment, because the bacteria transferred to the larvae hampered their development.

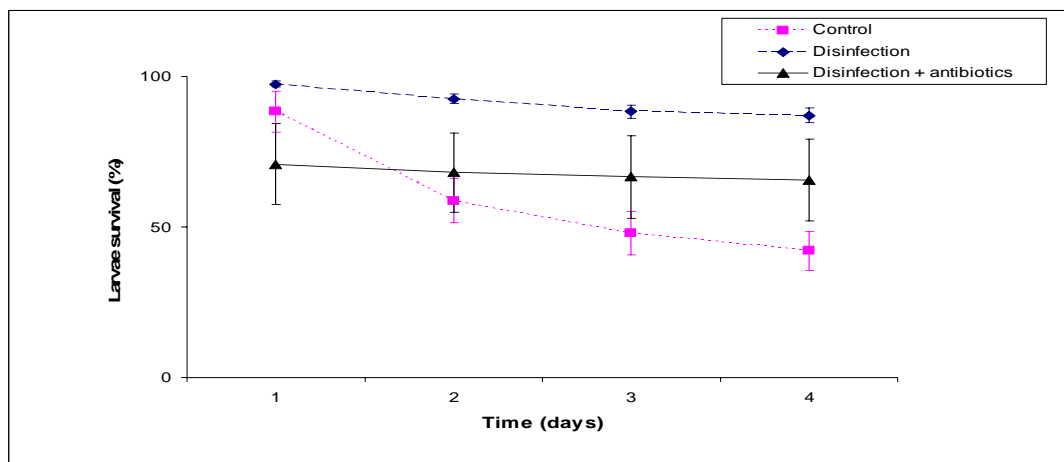


Fig. 12 Mean larvae survival \pm standard deviation of the larvae of experiment 1. The statistical analysis indicates no significant difference ($p>0,05$, Kruskal Wallis Test) between all treatments on day 1. On the day 2, 3 and 4, a significant difference ($p<0,05$) between the disinfection and the control was found.

4.4.2 Second experiment results

For a better visualisation, the graphics for the larval survival results were separated in 24h, 48h and 72h, respectively.

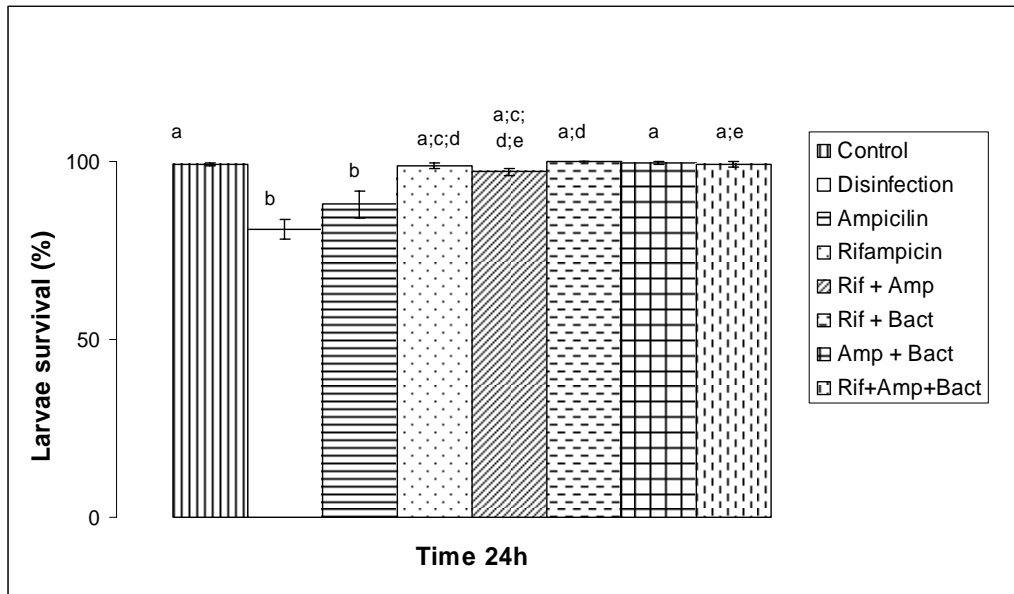


Fig. 13 Mean larvae survival \pm standard deviation of the larvae of experiment 2. Equal letters indicate no significant difference ($p > 0,05$; Kruskal Wallis Test) between the treatments.

The results for 24h (Fig. 13) of larvae survival showed no significant difference between the disinfection and the disinfection followed by the addition of ampicillin. However a significant difference was obtained between these two treatments and the others.

It is important remember that in all the treatments the eggs were first disinfected with glutaraldehyde, except control.

The disinfection treatment decreased the number of bacteria in comparison with the control on the first day (Table 3). On the second day, the bacteria count was still low, but there was an increase up to the hatching day, which occurred after four to five days of egg incubation (Fig. 4).

Some microorganisms are opportunistic pathogens and are a part of the normal flora in healthy hosts. In a stressed animal, these same bacteria may overcome the host defence mechanisms and cause morbidity or infection in the animal (Buller, 2004). The disinfection treatment, on the arrival day, probably kills the majority of the bacteria from the egg surface, unbalancing the natural flora, allowing the opportunistic pathogens to grow. Even the transfer of the hatched larvae to another erlenmeyer may carry over a big number of these bacteria that hamper the normal development of the larvae in the disinfection treatment (Fig. 13, 14 and 15).

According to Reitan *et al.* (1998), fish larvae drink water at an early developmental stage and the authors had measured the drinking rate of turbot larvae from day 2 after hatching and found as ingestion of 14 nl/h per larvae. This fact favours the opportunistic pathogenic bacteria to colonize the gut of larvae fish. As soon as they hatch, marine fish larvae ingest substantial numbers of bacteria through the intake of seawater for osmoregulation.

In the ampicillin treatment, probably because of the large number of bacteria resistant to this antibiotic, the antibiotic was not efficient to kill the opportunistic pathogenic bacteria. No adverse effects caused by the opportunistic pathogenic bacteria, were found in treatments with rifampicin and treatments with addition of LVS3-RP4 transconjugant. The rifampicin probably kills all the bacteria present avoiding any pathogenic effect on the larvae and the LVS3-RP4 transconjugant may have some probiotic effect against these bacteria or a direct positive effect on the larvae

One of the health benefits from consuming probiotics was attributed to their ability to provide protection against pathogens (Ray, 1996). Adhesion to and colonization of the mucosal surfaces are possible protective mechanisms through competition for binding sites and nutrients between the ingested microorganisms and potential pathogens (Salminen *et al.*, 1998; Westerdahl *et al.*, 1991).

On the second day of the larval survival experiment (Fig. 14), in three of the eight beakers from the “rifampicin with ampicillin with LVS3-RP4” high mortality was observed resulting in a big standard deviation for this treatment. The other five beakers within this treatment have 100% survival for the first and second day (Fig. 14). This

mortality may have been related to the fact that the beakers containing rifampicin in addition to ampicillin and bacteria were the last to receive the fish larvae from the bottles. The difference of time was no longer than 5 hours between the treatment with the abnormal result and the others treatments with addition of antibiotics and LVS3-RP4.

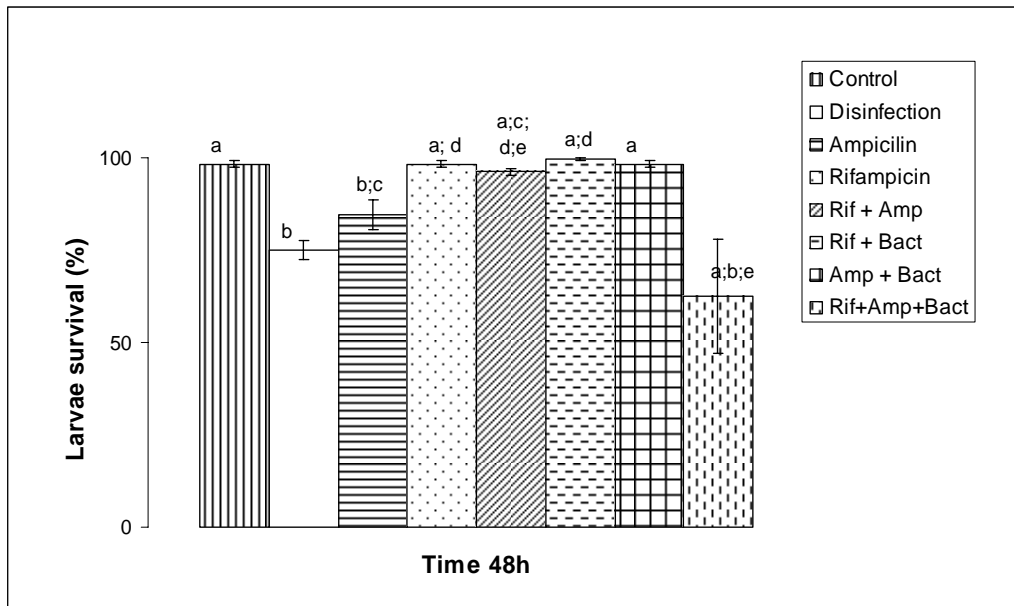


Fig. 14 Mean larvae survival \pm standard deviation of the larvae of experiment 2. The letters indicate no significant difference ($p > 0,05$; Kruskal Wallis Test) between the treatments.

On the last day of the larval survival experiment (Fig. 15), the disinfection treatment showed a cumulative mortality of 51,7%, disagreeing with others studies (Skjermo and Vadstein, 1999; Salvesen and Vadstein, 1995). This low survival may be attributed to the development of the opportunistic pathogenic bacteria.

The treatments with rifampicin and/or LVS3-RP4 were no significant different from the control, probably because the good quality eggs used in this experiment.

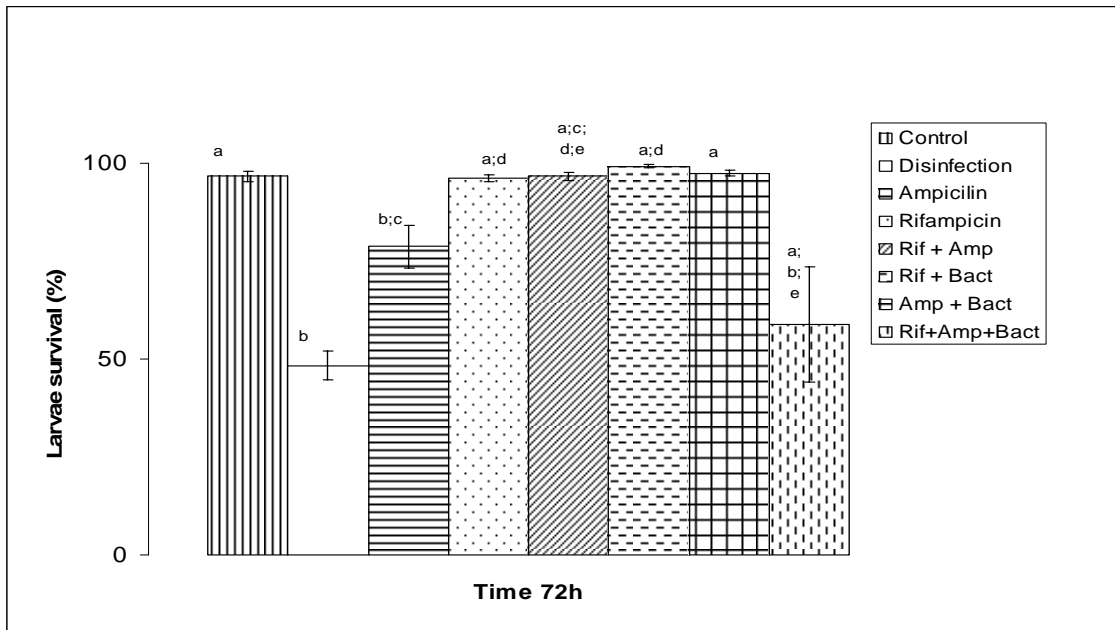


Fig. 15 Mean larvae survival \pm standard deviation of the larvae of experiment 2. The letters indicate no significant difference ($p > 0,05$; Kruskal Wallis Test) between the treatments.

4.5 Bacterial development

The aim of the next experiments was to understand more about the probably entrance of the LVS3-RP4 in the VBNC state and to understand more about the behaviour of this bacterium under the following different condition:

- different exposure time of resting cells into seawater at 16°C and 28°C;
- different incubation temperature after plating;
- different culture media and different concentration;
- different exposure time of resting cells into seawater with antibiotics at 16°C and 28°C.

4.5.1 Colony formation rate (days)

Two experiments were done to clarify the behaviour of the LVS3-RP4 transconjugant. Seawater supplemented with 10^5 CFU/ml, from colonies grown at 28°C in MA, was kept at 28°C and afterwards the cells were plated for single colonies at 28°C. The other experiment was done with colonies grown at 16°C in MA and

transferred, at 10^5 CFU/ml into seawater and kept at 16°C . Afterwards the plating was done at 16°C or 28°C .

A density of 10^5 CFU/ml of LVS3-RP4 transconjugant was added in this experiment but after 48h a density close to $1,5 \times 10^4$ CFU/ml was found (plates incubated for one day) (Fig. 16). This seems to indicate that not all bacteria added to the seawater were forming colonies. This effect happened after 48h of incubation in seawater. Further incubation in seawater (72h, 96h, 120h) does not change the detected amount of CFU/ml. However in all the cases, where the plates were further incubated at 28°C , more colonies could be detected. The amount of apparent CFU increased from $1,5 \times 10^4$ CFU/ml to $2,5 \times 10^4$ CFU/ml. The fact that not all added bacteria could be found back, raised the hypothesis that the bacteria were entering in a VBNC state. However mortality during the first 48h could account for the observed difference.

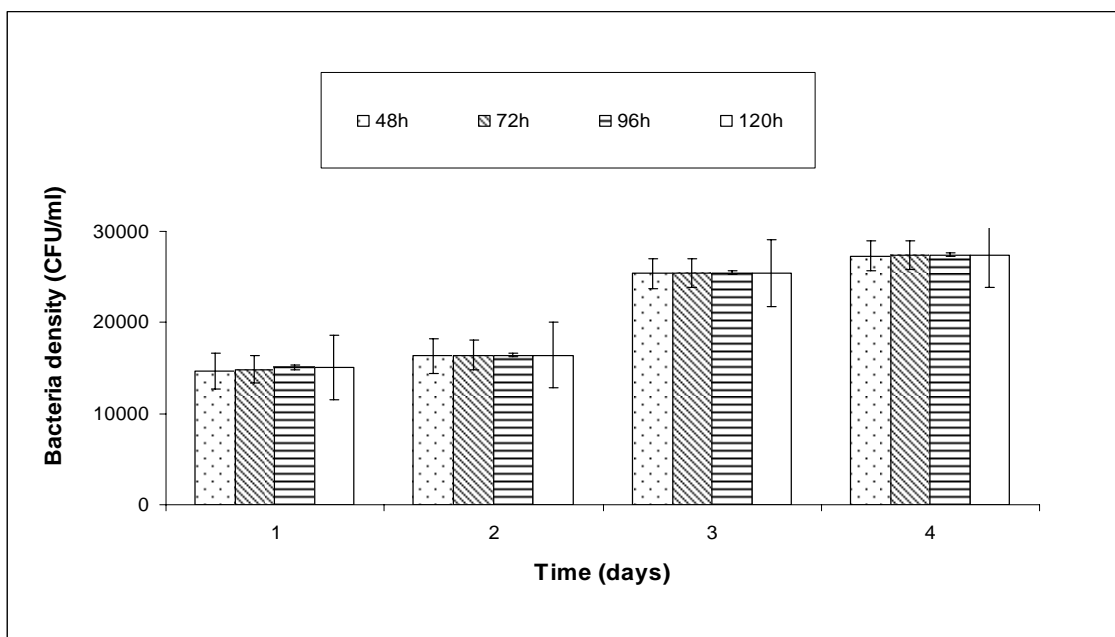


Fig. 16 Mean bacterial counts in CFU/ml \pm standard deviation. Plating efficiency of LVS3-RP4 grown at 28°C and transferred (10^5 CFU/ml) into seawater at 28°C and plated on MA (incubated at 28°C) was evaluated. Effect of incubation time in seawater (48, 72, 96, 120h). After plating, the colonies were counted after 1, 2, 3, 4 days (x-axis)

The second experiment on bacterial development was done with bacteria grown at 16°C in MA and transferred (10^5 CFU/ml) into seawater at 16°C . The transferred bacteria were verified for their ability to make colonies on MA after 48h, 72h, 96h and

120h. Plates were incubated at 28°C or 16°C and CFU/ml were counted after 1, 2, 3 and 4 days.

After 48h in seawater at 16°C, only $0,8 \times 10^4$ CFU/ml were detected by plating at 28°C. Further incubation at 16°C (72h, 96h, 120h) could not influence the observed amount of CFU. In contrast to the previous experiment, the amount of CFU detected was not influenced by the plate incubation time (Fig. 17).

No colony was found when the plates were incubated at 16°C. The plates were observed for 15 days. This could be an evidence of the LVS3-RP4 transconjugant entering in VBNC state.

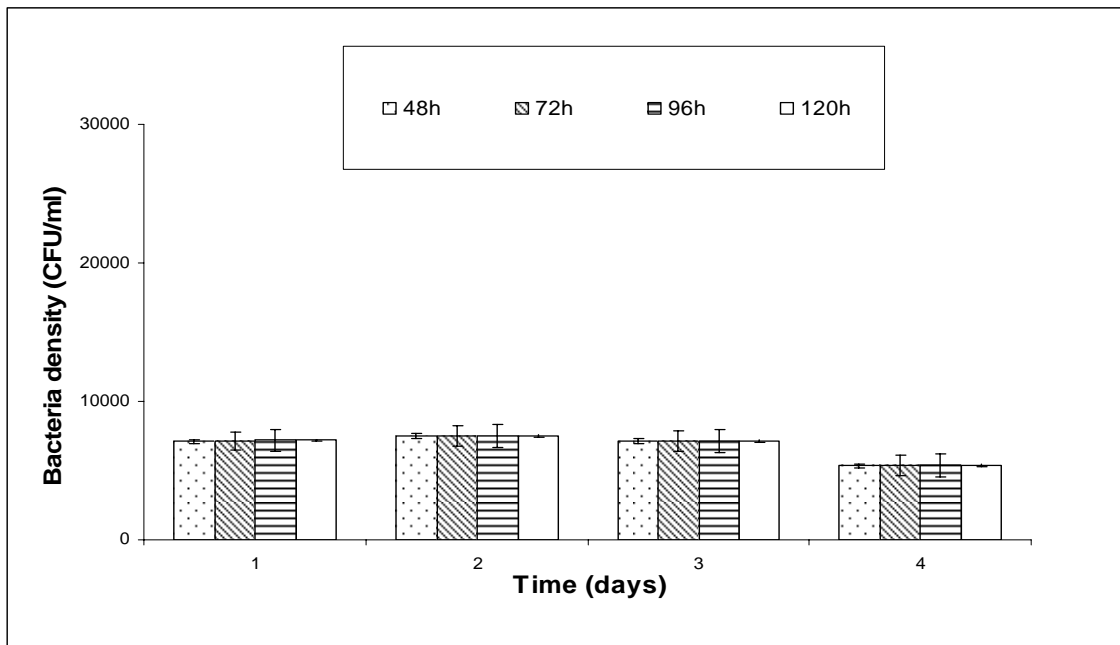


Fig. 17 Mean bacterial counts in CFU/ml \pm standard deviation. Plating efficiency of LVS3-RP4 grown at 16°C, transferred (10^5 CFU/ml) into seawater at 16°C and plated on MA (incubated at 28°C) was evaluated. Effect of incubation time in seawater (48, 72, 96, 120h). After plating, the colonies were counted after 1, 2, 3, 4 days (x-axis).

4.5.2 Colony formation rate (hours)

The LVS3-RP4 transconjugant ability to make colonies was tested after 30min, 1h, 2h, 4h and 8h of storage at 28°C or 16°C. The bacteria were previously grown at

28°C on MA and transferred to seawater for the different treatments. The CFU/ml counts were observed after 48h, 72h, 96h and 120h.

In one treatment (Fig. 18), the bacteria grown at 28°C were transferred (10^5 CFU/ml) into seawater. The seawater was kept at 28°C and plated on MA after 30min, 1h, 2h, 4h and 8h after the transfer. The incubation temperature was 28°C and the colonies forming units were counted after 48h, 72h, 96h and 120h. In the other treatment, the bacteria grown at 28°C and afterwards transferred (10^5 CFU/ml) into seawater. The seawater was kept at 16°C and the bacteria were plated on MA after 30min, 1h, 2h, 4h and 8h after the transfer. The incubation temperature was 16°C and the colonies forming units were verified after 15 days.

The capacity to make colonies stayed constant over the experimental period when all handlings were performed isothermic at 28°C. (Fig. 18). The amount of bacteria detected (1.5×10^4 CFU/ml) was lower than 10^5 CFU/ml like in the previous experiment (Fig. 16).

The treatment using bacteria grown at 28°C, transferred into seawater at 16°C and, after the storage period (8h), plated and incubated at 16°C was observed for 15 days. No colony was detected on plates in this period. The results show a possible viable but non-culturable state of LVS3-RP4 in our study.

According to Maalej *et al.* (2004), *A. hydrophila*, collected in an exponential growth phase and incubated at 5°C in sterile seawater, were viable but nonculturable state on day 53, when culturable cells reached a level below 0,1 CFU/ml, in their study.

Aeromonas sp. in the marine waters have high numbers of culturable cells noticed in late summer/early autumn, when the temperatures were around 23°C, this is probably due to the prolonged survival in cold water of injured cells at very low levels and subsequent regrowth when temperature becomes favourable (Maalej *et al.*, 2003).

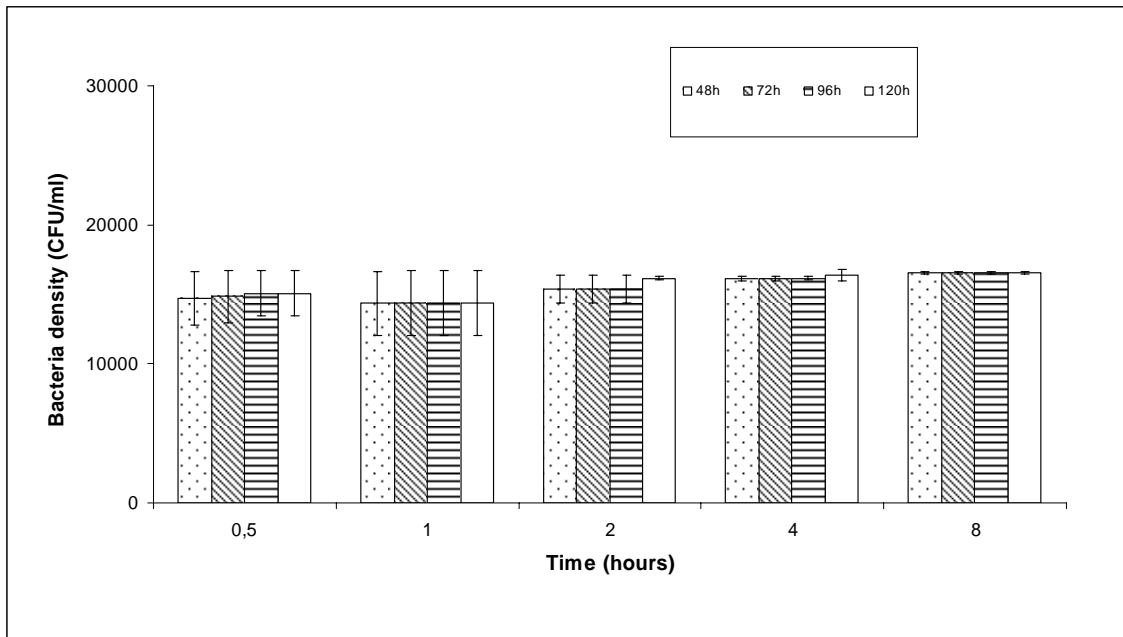


Fig. 18 Mean bacterial counts in CFU/ml \pm standard deviation. Plating efficiency of LVS3-RP4 grown at 28°C, transferred (10^5 CFU/ml) into seawater at 28°C and plated on MA (incubated at 28°C) was evaluated. Effect of incubation time in seawater (0,5h, 1h, 2h, 4h and 8h). After plating, the colonies were counted after 48h, 72h, 96h and 120h (x-axis).

4.5.3 Plating efficiency of LVS3-RP4 transconjugant after exposure to different antibiotics

Two different experiments were conducted to check the LVS3-RP4 ability to make colonies by plating. In all of the following experiments, the bacteria were grown at 28°C and afterwards transferred into seawater according with the experiments. The antibiotics were added at 10mg/l each into the seawater.

In the first experiment, the seawater was kept for one day or two days at 16°C before plating. The bacteria were plated on MA (Fig. 19) or MA 10% (Fig. 20) and incubated at 28°C.

In the second experiment, seawater was kept for one day at 16°C and one day at 28°C, respectively or the seawater was kept for one day at 16°C and more two days at 28°C. After plating, the plates were incubated at 28°C. The plating was done on MA (Fig. 21) or MA 10% (Fig. 22).

In both experiments, in which resting cells were kept at 16°C for one day or two days, the results for LVS3-RP4 plate counts were very low compared to the added concentration 10⁵ CFU/ml of LVS3-RP4 at the beginning of the experiment (Fig. 19).

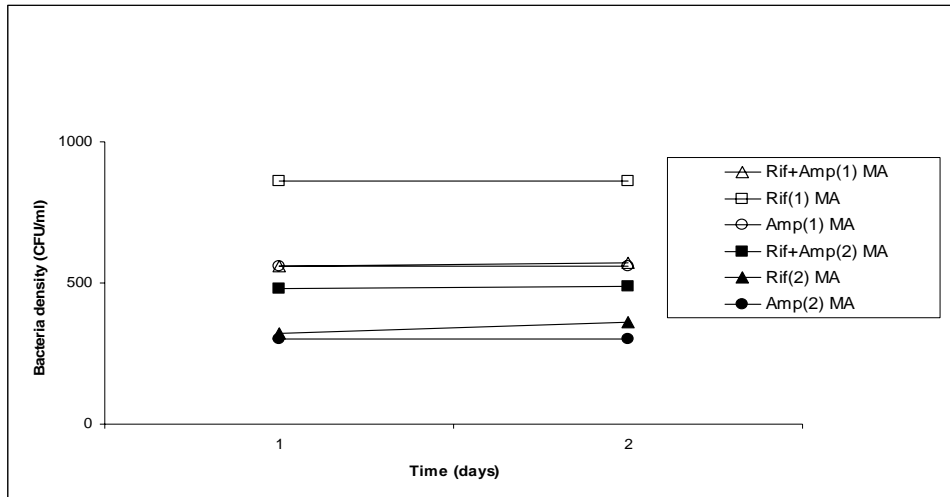


Fig. 19 Bacteria counts on MA. (1) Seawater with LVS3-RP4 (added at 10⁵ CFU/ml) and antibiotics was kept for one day at 16°C before plating; (2) Seawater with LVS3-RP4 (added at 10⁵ CFU/ml) and antibiotics was kept for two days at 16°C before plating; Plates were incubated at 28°C

In these experiments, the higher LVS3-RP4 counts were found in the treatments kept one day at 16°C (Fig. 19), and the lower counts were found for those treatments kept for two days at 16°C. This result indicates the probable entering of the LVS3-RP4 transconjugant in VBNC state.

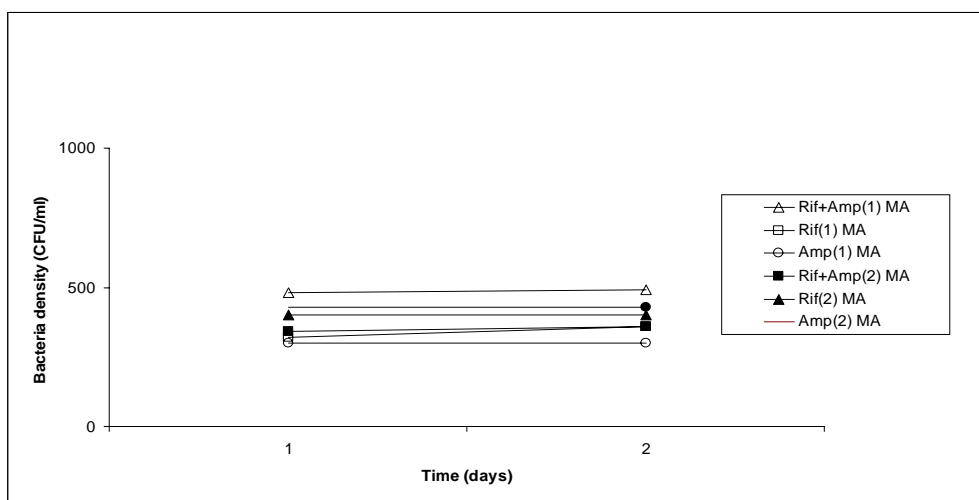


Fig. 20 Bacteria counts on MA 10%. (1) Seawater with LVS3-RP4 (added at 10⁵ CFU/ml) and antibiotics was kept for one day at 16°C before plating; (2) Seawater with LVS3-RP4 (added at 10⁵ CFU/ml) and antibiotics was kept for two days at 16°C before plating; Plates were incubated at 28°C

No major differences were found in these two experiments. Very similar plate counts were found when resting cells (10^5 CFU/ml) were incubated in seawater in the presence of antibiotics for one or two days.

In a subsequent experiment, MA10% was used to check if the bacteria can grow better in low nutrient media in comparison with normal MA. The plate count was lower in MA 10% (Fig. 20), indicative that MA 10% did not promote colony formation for LVS3-RP4. The bacterium prefers the normal amount of nutrient available in MA (Fig. 19).

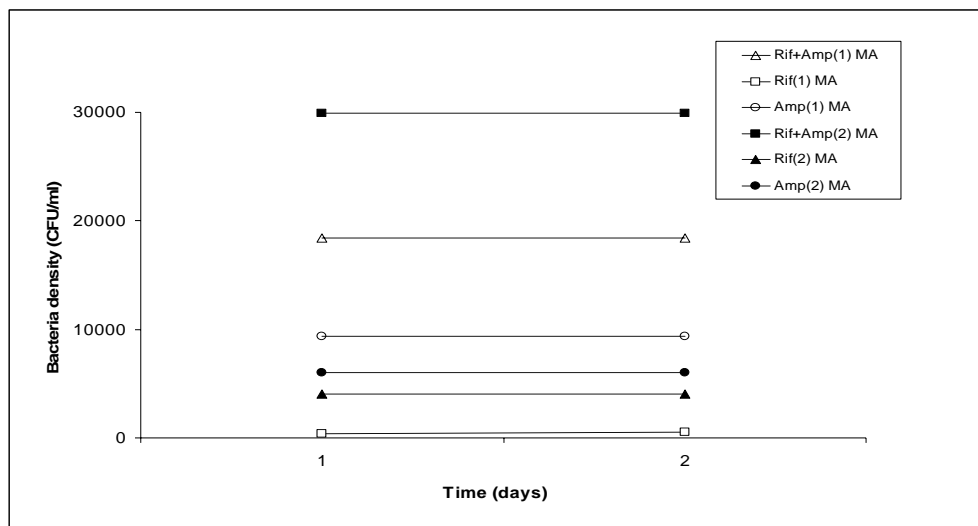


Fig. 21 Bacteria counts on MA. (1) Seawater with LVS3-RP4 (added at 10^5 CFU/ml) and antibiotics was kept for one day at 16°C and one day at 28°C before plating; (2) Seawater with LVS3-RP4 (added at 10^5 CFU/ml) and antibiotics was kept for one days at 16°C and two days at 28°C before plating; Plates were incubated at 28°C

In this experiment resting cells of LVS3-RP4 (10^5 CFU/ml added to seawater containing antibiotics) were kept one day at 16°C and one at 28°C or one day at 16°C and two days at 28°C (Fig. 21). When plated, these solutions contained a higher amount of CFU in relation to the experiments shown in Fig 19 and 20. Yet there was still an underestimation. The highest plate count ($2,9 \times 10^4$ CFU/ml) was obtained for resting cells exposed to “rifampicin with ampicillin” and kept for one day at 16°C and two days at 28°C . The experiments shown in Fig. 19 till Fig. 22 seems to indicated that resting cells enter the VBNC state by incubating them at 16°C but that they can leave this state by increasing the temperature to 28°C before plating.

In three of four results (Fig. 20, 21 and 22), the highest plates count were obtained with resting cells exposed to rifampicin with ampicillin. This treatment probably provides a better condition (e.g. pH) to the LVS3-RP4 transconjugant, delaying the entering of this bacterium into VBNC state and providing a higher plating efficiency.

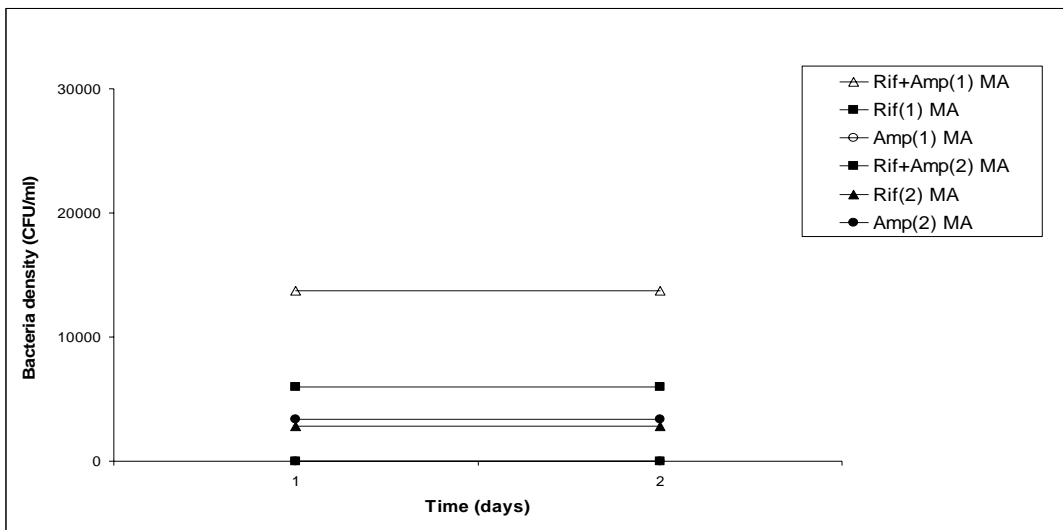


Fig. 22 Bacteria counts on MA 10%. (1) Seawater with LVS3-RP4 (added at 10^5 CFU/ml) and antibiotics was kept for one day at 16°C and one day at 28°C before plating; (2) Seawater with LVS3-RP4 (added at 10^5 CFU/ml) and antibiotics was kept for one days at 16°C and two days at 28°C before plating; Plates incubated at 28°C

The MA 10% showed a lower count of bacteria differing from the normal MA. This result showed a preference of this bacterium for the MA media (Fig. 22).

The Fig. 22, showed a higher density of colony in the “rifampicin with ampicillin” treatment kept one day at 16°C and one day at 28°C . The others treatments showed an expected result where the treatment kept for one day at 16°C and two at 28°C should show higher amounts of CFU/ml. The samples were conducted without replicates in these experiments therefore this result should be taken with caution.

4.5.4 Qualitative experiment with different media and the LVS3-RP4 transconjugant

In this experiment were used turbot larvae from the second turbot experiment as putative source for LVS3 bacteria. The larvae were homogenized in the different media with a drop of seawater.

The results (Table 5) showed the presence of the LVS3-RP4 transconjugant bacteria in all treatments where they were previously added “rif + bact”, “amp + bact.” and “rif + amp + bact”. This means that the LVS3-RP4 transconjugant were present until the end of the turbot experiment, but the amounts of bacteria probably were not sufficiently to express high positive effect on the turbot development.

Bacteria were found in the “control”, “disinfection”, “disinfection with ampicillin”, “disinfection with rifampicin” treatments and could be handling contamination or bacteria that were able to survive through the disinfection treatments. The results indicate rifampicin with ampicillin as the most efficient disinfection treatment (Table 5).

Table 5. Qualitative results of plating on different culture media. Turbot larvae at the end of the turbot experiment and a drop of seawater were homogenized on different media; Yes: presence of CFU; No: no CFU. The plates were incubated at 28°C and verified after 48h

MEDIA	Control	Disinf.	Amp.	Rif. +		Amp. +		Rif. + Amp. +
				Rif.	Amp.	Bact.	Bact.	Bact.
Fish Agar	Yes	Yes	No	No	Yes	Yes	Yes	Yes
Fish Agar	No	Yes	No	No	No	Yes	Yes	Yes
MA	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes
MA 1%	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes
MA 1%	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes
MA 10%	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes
MA 10%	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes
MA (Rif+Amp)	Yes	Yes	No	No	No	Yes	Yes	Yes
MA (Rif+Amp)	Yes	Yes	No	No	No	Yes	Yes	Yes

5 CONCLUSIONS

The results from this study allow the following conclusions:

The efficacy of plasmid transfer between *Escherichia coli* (RP4) and *Aeromonas hydrophila* (LVS3) was estimated to be 6,58%.

The disinfection could not reduce the bacterial load of the eggs in case of poor egg quality.

The disinfection of turbot eggs with glutaraldehyde at 200 mg/l [50% (v/v) in water] for 5 minutes is not completed, allowing opportunistic pathogens to emerge and cause adverse effect to the larvae.

The disinfection of eggs of poor quality with glutaraldehyde has subsequently a positive effect on larval survival.

Tetracycline and/or kanamycin at a concentration of 10 mg/l each, caused teratogenic effect on turbot eggs (hatchability).

Rifampicin was an efficient disinfectant when used after glutaraldehyde, avoiding the bacterial pathogenic adverse effect on the turbot larvae.

The negative effect of bacteria in the larval survival could not be mitigated by ampicillin.

The LVS3-RP4 transconjugant has a probiotic effect as evidenced by a positive effect on the larval survival, despite entering into a viable but non-culturable (VBNC) state.

The LVS3-RP4 transconjugant start entering in the VBNC state at 16°C.

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APPENDICES

Appendix 1

1.1. NSS (1L distilled water)

NaCl (Sigma)- 17,6g	MgCl ₂ ·6H ₂ O (VWR)- 1,87g
Na ₂ SO ₄ (VWR) - 1,47g	CaCl ₂ ·2H ₂ O (VWR)- 0,41g
NaHCO ₃ (VWR) - 0,08g	SrCl ₂ ·6H ₂ O (VWR)– 0,008g
KCl (VWR)- 0,25g	H ₃ BO ₃ (Sigma)– 0,008g
KBr (VWR)- 0,04g	

1.2. Luria Bertani broth (LB) (1L distilled water)

Yeast Extract (Sigma) - 5g
Tryptone (Difco) - 10g
NaCl (Sigma) – 10g

1.3. Luria Bertani Agar (LBA) (1L distilled water)

Yeast Extract (Sigma) - 5g
Tryptone (Difco) - 10g
NaCl (Sigma) - 10g
Agar technical (Difco) - 18g

1.4. Fixation Liquid (1,8L distilled water)

NaH ₂ PO ₄ ·H ₂ O (VWR) – 8g
Na ₂ HPO ₄ (VWR) – 13g
Formaldehyde (35%, VWR) – 200ml

1.5. PBS – Buffer

NaCl (Sigma) – 8g
KCl (VWR) – 0,2g
Na ₂ HPO ₄ (VWR) – 1,44g
KH ₂ PO ₄ (VWR) – 0,24g

Appendix 2

2.1 Bacteria counts for conjugation efficiency experiment between *Aeromonas hydrophila* (LVS3) and *Escherichia coli* (RP4): CFU/ml on different culture media (at 28°C for 24h)

Mac Conkey	LBA (Rif. + Tet.)	LBA (Rif.)	LBA (Rif. + Kn.)	LBA (Rif. + Amp.)
11,7*10 ⁷	8,8*10 ⁶	9,8*10 ⁷	2,0*10 ⁷	10,6*10 ⁷

2.2 Hatching percentage (Experiment I)

Treatment	Control	Disinfection	Disinfection with antibiotics
Mean hatching %	15,8	11,4	3,3
Standard deviation %	±2,6	±5,9	±3,1
Coef. of variation %	0,1	0,5	0,9

2.3 Hatching percentage (Experiment II)

Egg hatchability	Control	Disinf.	Amp.	Rif.	Rif	Rif	Amp	Rif
					+	+	+	+Amp
					Amp	Bact	Bact	+Bact
Hatching %	115,6	160,0	173,3	168,9	146,7	191,1	151,1	106,7
Stand.Dev. %	±21,8	±58,4	±32,7	±46,7	±28,0	±81,6	±60,0	±29,2
Coef. of variation %	0,2	0,4	0,2	0,3	0,2	0,4	0,4	0,3

2.4 Mean survival rate (in percentage) ± Standard deviations (Experiment I)

Treatment	Day 1	Day 2	Day 3	Day 4
Control	88,3 ± 6,8	58,7 ± 7,4	47,9 ± 7,2	42,0 ± 6,5
Disinfection	97,5 ± 1,0	92,5 ± 1,6	88,3 ± 2,2	87,0 ± 2,5
Disinfection with antibiotics	70,9 ± 13,6	68,1 ± 13,1	66,6 ± 13,8	65,7 ± 13,6

2.5 Mean survival rate (in percentage) ± Standard deviations (Experiment II)

Treatment	Day 1	Day 2	Day 3
Control	99,2 ± 0,5	98,3 ± 0,8	96,7 ± 1,4
Disinf.	80,8 ± 2,7	75,0 ± 2,4	48,3 ± 3,6
Amp.	87,9 ± 3,7	84,6 ± 4,0	78,8 ± 5,4
Rif.	98,8 ± 0,7	98,3 ± 0,8	96,3 ± 0,8
Rif + Amp	97,1 ± 1,1	96,3 ± 1,0	96,7 ± 1,1
Rif + Bact	100 ± 0,0	99,6 ± 0,3	99,2 ± 0,5
Amp + Bact	99,6 ± 0,4	98,3 ± 1,1	97,5 ± 0,7
Rif+Amp+Bact	99,2 ± 0,7	62,5 ± 15,5	58,8 ± 14,7

2.6 Mean bacterial counts in CFU/ml \pm standard deviation. Plating efficiency experiment (Fig. 16). Colony formation rate (days)

	48h	Stan.Dev.48h	72h	Stan.Dev.72h	96h	Stan.Dev.96h	120h	Stan.Dev.120h
Day 1	14,7*10 ³	$\pm 1,9*10^3$	14,8*10 ³	$\pm 1,9*10^3$	15*10 ³	$\pm 1,6*10^3$	15*10 ³	$\pm 1,6*10^3$
Day 2	16,3*10 ³	$\pm 1,5*10^3$	16,4*10 ³	$\pm 1,6*10^3$	16,4*10 ³	$\pm 1,6*10^3$	16,4*10 ³	$\pm 1,6*10^3$
Day 3	25,4*10 ³	$\pm 2,4*10^2$	25,4*10 ³	$\pm 1,9*10^2$	25,5*10 ³	$\pm 2,1*10^2$	25,5*10 ³	$\pm 2,1*10^2$
Day 4	27,3*10 ³	$\pm 3,5*10^3$	27,4*10 ³	$\pm 3,6*10^3$	27,4*10 ³	$\pm 3,6*10^3$	27,4*10 ³	$\pm 3,6*10^3$

2.7 Mean bacterial counts in CFU/ml \pm standard deviation. Plating efficiency experiment (Fig. 17). Colony formation rate (days)

	48h	Stan.Dev.48h	72h	Stan.Dev.72h	96h	Stan.Dev.96h	120h	Stan.Dev.120h
Day 1	7,1*10 ³	$\pm 1,4*10^2$	7,1*10 ³	$\pm 1,8*10^2$	7,2*10 ³	$\pm 1,4*10^2$	7,2*10 ³	± 129
Day 2	7,5*10 ³	$\pm 6,8*10^2$	7,5*10 ³	$\pm 7,0*10^2$	7,5*10 ³	$\pm 7,0*10^2$	7,5*10 ³	± 707
Day 3	7,1*10 ³	$\pm 8,0*10^2$	7,1*10 ³	$\pm 8,2*10^2$	7,1*10 ³	$\pm 8,2*10^2$	7,1*10 ³	± 820
Day 4	5,3*10 ³	$\pm 8,5*10^2$	5,4*10 ³	$\pm 1,3*10^2$	5,4*10 ³	$\pm 1,3*10^2$	5,4*10 ³	± 113

2.8 Mean bacterial counts in CFU/ml \pm standard deviation. Plating efficiency experiment (Fig. 18). Colony formation rate (hours)

	48h	Stan.Dev.30min	72h	Stan.Dev.1h	96h	Stan.Dev.2h	120h	Stan.Dev.120h
0,5h	14,7*10 ³	$\pm 1,9*10^3$	14,8*10 ³	$\pm 1,9*10^3$	15*10 ³	$\pm 1,6*10^3$	15*10 ³	$\pm 1,6*10^3$
1h	14,3*10 ³	$\pm 2,3*10^3$	14,3*10 ³	$\pm 2,3*10^3$	14,4*10 ³	$\pm 2,3*10^3$	14,4*10 ³	$\pm 2,3*10^3$
2h	15,4*10 ³	$\pm 9,7*10^2$	15,4*10 ³	$\pm 9,7*10^2$	15,4*10 ³	$\pm 1,0*10^3$	16,1*10 ³	$\pm 1,3*10^2$
4h	16,1*10 ³	$\pm 1,8*10^2$	16,1*10 ³	$\pm 1,6*10^2$	16,1*10 ³	$\pm 1,3*10^2$	16,4*10 ³	$\pm 4,1*10^2$
8h	16,5*10 ³	$\pm 9,9*10^1$	16,5*10 ³	$\pm 8,5*10^1$	16,5*10 ³	$\pm 8,5*10^1$	16,5*10 ³	$\pm 8,5*10^1$

2.9 Bacteria count on MA. Plating efficiency with antibiotics (Fig. 19)

MA (1)	24h	48h
Rif+ Amp	5,6*10 ³	5,7*10 ³
Rif	8,6*10 ³	8,6*10 ³
Amp	5,6*10 ³	5,6*10 ³
MA (2)	24h	48h
Rif+ Amp	4,8*10 ³	4,9*10 ³
Rif	3,2*10 ³	3,6*10 ³
Amp	3,0*10 ³	3,0*10 ³

2.10 Bacteria count on MA 10% . Plating efficiency with antibiotics (Fig. 20)

MA 10% (1)	24h	48h
Rif+ Amp	4,6*10 ³	4,7*10 ³
Rif	3,2*10 ³	3,4*10 ³
Amp	2,8*10 ³	2,8*10 ³
MA 10% (2)	24h	48h
Rif+ Amp	3,4*10 ³	360
Rif	4,0*10 ³	4,0*10 ³
Amp	4,3*10 ³	4,3*10 ³

2.11 Bacteria count on MA. Plating efficiency with antibiotics (Fig. 21)

MA (1)	24h	48h
Rif+ Amp	$1,8 \cdot 10^4$	$1,8 \cdot 10^4$
Rif	$4,0 \cdot 10^2$	$5,4 \cdot 10^2$
Amp	$9,4 \cdot 10^3$	$9,4 \cdot 10^3$
MA (2)	24h	48h
Rif+ Amp	$6,0 \cdot 10^3$	$6,0 \cdot 10^3$
Rif	$4,0 \cdot 10^3$	$4,0 \cdot 10^3$
Amp	$2,9 \cdot 10^4$	$2,9 \cdot 10^4$

2.12 Bacteria count on MA 10%. Plating efficiency with antibiotics (Fig. 22)

MA 10% (1)	24h	48h
Rif+ Amp	$1,4 \cdot 10^4$	$1,4 \cdot 10^4$
Rif	0	0
Amp	0	$2,0 \cdot 10^1$
MA 10% (2)	24h	48h
Rif+ Amp	$6,0 \cdot 10^3$	$6,0 \cdot 10^3$
Rif	$2,8 \cdot 10^3$	$2,8 \cdot 10^3$
Amp	$3,3 \cdot 10^3$	$3,3 \cdot 10^3$