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Immobilization of *Phaeobacter* 27-4 in biofilters as a strategy for the control of *Vibrionaceae* infections in marine fish larval rearing

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ABSTRACT

The purpose of this Ph.D. Thesis was to study the immobilization of the probiotic strain *Phaeobacter* 27-4 in biofilters as a new strategy for the control of *Vibrionaceae* infections in turbot (*Psetta maxima*) larval rearing. This new strategy guarantees the permanence of the probiotic bacteria in the rearing system and protects turbot larvae against the fish pathogen *Listonella anguillarum* 90-11-287 at the beginning of exogenous feeding on the rotifer *Brachionus plicatilis*.

As a first step to accomplish the objectives, a reproducible and specific real-time PCR method was developed for detection and quantification of the fish probiotic *Phaeobacter* 27-4 and the target pathogens *L. anguillarum* 90-11-287 and *V. splendidus* DMC-1 in presence of all the organisms involved in turbot larval rearing: microalgae (*Isochrysis galbana*), rotifers (*Brachionus plicatilis*), *Artemia* nauplii and turbot larvae. The developed real-time PCR protocol allowed monitoring and quantification of probiotic and pathogenic bacteria during *in vivo* trials.

As rotifer is the usual way of entry for potential pathogenic *Vibrionaceae* into fish larvae, in a second step in this Ph.D. Thesis, the colonization and residence time of two pathogenic *Vibrionaceae* strains (*L. anguillarum* 90-11-287 and *V. splendidus* DMC-1) in rotifers were studied. *L. anguillarum* colonized rotifers more efficiently than *V. splendidus* and both pathogenic strains were released from rotifers to seawater, after infected rotifers were transferred to rearing tanks. Residence time of *L. anguillarum* was longer than for *V. splendidus*, being released slower to surrounding seawater. *V. splendidus* grew and became predominant in the seawater of tanks. Both pathogens remained in rotifer or seawater enough time to infect fish larvae, but their different behaviour could determine different infection patterns, preferentially by ingestion of prey or by active intake or contact with surrounding seawater.

The effect of *L. anguillarum* and *V. splendidus* on the bacterial community associated with rotifers and seawater of rearing tanks was analysed by DGGE of PCR-amplified 16S rDNA fragments. The bacterial community of rotifers did not present marked species dominance, being composed by Gram negative bacteria belonging to α-Proteobacteria, γ-Proteobacteria, *Cytophaga-Flexibacter-Bacteroides* group and a Gram positive bacterium (*Microbacterium* sp). The incorporation of *L. anguillarum* or *V. splendidus* did not reduce bacterial diversity and shifts in bacteria populations could be explained by bacterial exchange between rotifers and seawater.

The third step consisted in the application of biofilters with the probiotic strain *Phaeobacter* 27-4 against *Vibrionaceae* infections in the rearing of turbot larvae. The growth and the antagonism (*in vivo* and *in vitro*) of the probiotic strain *Phaeobacter* 27-4 was tested with different supports used in aquaculture biofilters (plastic balls, sintered glass pellets and ceramic cylinders) and under different culture conditions (soaked stagnant and submerged with or without agitation). The attachment and growth of *Phaeobacter*, as well as the formation of rosette-shaped microcolonies and the subsequent development of a biofilm, were different depending on the support and on the culture conditions. A multilayer biofilm was only detected on ceramic cylinders cultured in submerged stagnant conditions.

The *in vitro* antagonistic activity of *Phaeobacter* 27-4 biofilters against the pathogenic *Vibrionaceae L. anguillarum* 90-11-287 and *V. splendidus* DMC-1 was different when immobilized on biofilters made of different materials. The degree of antagonism was affected by the support and culture conditions used for the growth of the probiotic strain. In absence of nutrients, the inactivation was similar for both pathogens, but the inhibition of growth promoted by the probiotic, in presence of nutrients, was higher for *L. anguillarum* than for *V. splendidus*. In presence of *Phaeobacter*, *V. splendidus* grew more than *L. anguillarum* and induced a lower growth of the probiotic in the culture medium. The presence of *Phaeobacter* also diminished the attachment of both pathogens to ceramic and sintered glass but not to plastic supports.

The permanence and detachment kinetic of *Phaeobacter* 27-4 was tested in tanks with green seawater maintained under the conditions used in larval rearing. *Phaeobacter* showed a better permanence in porous supports, compared with the plastic one, being the lowest slope of detachment registered in ceramic biofilters. In DGGE profiles, ceramic biofilters showed the lowest number of bands, indicating that the presence of *Phaeobacter* in those biofilters avoids the colonization by other bacteria.

Ceramic cylinders were selected for preparation of biofilters to be used in challenge trials at pilot-scale due to the capability of *Phaeobacter* 27-4 in that support to: *i*) develop a biofilm formed by rosette-shaped microcolonies, *ii*) antagonize two pathogens (*L. anguillarum* and *V. splendidus*), and *iii*) remain longer in the biofilters when maintained under larval rearing conditions. Additionally, ceramic is a resistant material, which would be advantageous for the scale-up in aquaculture facilities.

In small scale trials conducted under larval rearing conditions and different nutrients levels, matured *Phaeobacter* 27-4 ceramic biofilters reduced the concentration of *L. anguillarum* 90-11-287 and total *Vibrionaceae* in green seawater with different nutrients levels.

Phaeobacter 27-4 biofilters significantly reduced a 45 % the accumulated mortality in turbot larvae infected with the pathogen *L. anguillarum* 90-11-287 by diminishing pathogen levels and the concentration of total *Vibrionaceae*, mainly in seawater. Additionally, the probiotic biofilter reduced turbidity in the rearing tanks.

DGGE analysis revealed that the incorporation of *L. anguillarum* or *Phaeobacter* matured biofilters did not displace or modify significantly the bacterial microbiota present on larvae, which showed low bacterial diversity. However, the presence of *Phaeobacter* biofilters diminished the carrying capacity in seawater of rearing tanks. The shifts occurring in the bacterial community of turbot larvae reflected the influence of rotifers on larvae bacterial communities, whereas in the seawater shifts were marked by disappearance of bands when *Phaeobacter* biofilters were present.

Biofilters acted as a probiotic reservoir in the rearing system, maintaining the level of the probiotic in the seawater of tanks for at least ten days, period in which larvae are fed on rotifers and which is considered critical for larval survival. An important advantage of this biofilters is that their use does not require repeated additions of the probiotic, avoiding long-term cultures and bioencapsulation in rotifers. This feature simplifies the potential transference of this methodology to industrial hatcheries.

1

INTRODUCTION

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1. INTRODUCTION

New strategies for the control of bacterial infections in marine fish larval rearing

One of the limiting steps of marine fish aquaculture expansion is the supply of juvenile fish. Intensive fish larvae production is highly susceptible to the proliferation of bacteria, which may cause poor growth or mass mortality of the larvae. In most cases, mortality cannot be attributed to a specific pathogen, but to the proliferation of opportunistic bacteria (Olafsen, 1993, 2001). The rearing environment, with high larvae densities and high load of organic matter (from faeces, dead larvae debris or from live feed) is highly susceptible for bacterial growth. The control of bacteria in rearing systems and live feed production is a determinant factor for survival of fish larvae (Dhert *et al.*, 2001; Planas and Cunha, 1999; Skjermo and Vadstein, 1999) and the increment in the survival of larvae treated with antibiotics supports this fact (Gatesoupe, 1982, 1989; Pérez-Benavente and Gatesoupe, 1988).

In aquatic environment, the use of antibiotics induces the development of resistances, which can be transferred to other bacteria, including pathogenic strains to fish or even humans (Cabello, 2006). Therefore, the use of antibiotics should be kept to a minimum. Vaccination of juveniles has drastically reduced the use of chemotherapeutics in aquaculture, increasing the survival of farmed fish. However, fish larvae have an undeveloped immune system, relying on maternal antibodies and non specific immune response (Vadstein, 1997), which prevents the use of vaccination.

Usual approaches to control bacterial growth in intensive rearing of marine fish larvae are based on preventive hygienic measures, leading to the development of a clean environment, by seawater treatment processes (e.g. filtration, UV-irradiation, ozonization, disinfectants). Disinfection of fish eggs (Salvesen and Vadstein, 1995) and measures for control of bacterial microbiota in live feed such as, disinfection of rotifer eggs for the production of axenic cultures (Dhert *et al.*, 2001; Douillet, 1998), treatment with hydrogen peroxide (Giménez *et al.*, 2006) or ultraviolet radiation for partial decontamination (Munro *et al.*, 1993, 1999) have been proposed but would be difficult to implement at industrial scale. Complete elimination of bacteria from the organisms and culture system is not possible, while disinfection implies in most cases the loss of a

stable microbial population, with dominance of slow-growing bacteria (*K* strategists), which may promote, in a nutrient-rich environment (due to feed and excretions) as are larval culture systems, the rapid colonization by opportunistic bacteria (*r* strategists), with high growth rates and potentially harmful to fish larvae (Skjermo and Vadstein, 1993).

Therefore, at present, efforts have turned to new strategies for the control of bacterial microbiota in the rearing systems that, avoiding the use of antibiotics and disinfectants, would conduct to a more environmentally friendly and sustainable aquaculture. The approach would be in accordance with the Ecosystem Approach for the sustainable growth and expansion of aquaculture, promoted by FAO in *The State of the World Fisheries and Aquaculture* (FAO 2006, pp. 76-84).

This chapter discusses these new strategies. Most of them have been proposed or developed at laboratory scale and some of them in pilot-plant trials. Only a few of them are being employed at an industrial scale.

1.1. Bacteria in fish larvae

Fish larvae at hatching are commonly colonized by very few bacteria, which may originate from the egg surface, or from the surrounding seawater. During the yolk-sac stage, marine fish larvae drink seawater to osmoregulate. They thus accumulate bacteria and microalgae in their gut (Reitan *et al.*, 1998). The numbers of bacteria during this phase of rearing are low (< 100 CFU per larva), whereas the biodiversity of microbiota can be quite high (Hansen and Olafsen, 1999).

As soon as fish larvae start to capture and ingest live prey, the number of bacteria in the larvae increase exponentially up to values as high as 10^5 per larva within a few days (Munro *et al.*, 1994; Muroga *et al.*, 1987). Members of α- and γ-Proteobacteria are quite common in fish larvae (Nakase *et al.*, 2007; Schulze *et al.*, 2006). These bacteria are located mainly in the larval gut, as proven in the cases where disinfection of the surface of the larvae does not decrease to a noticeable degree the numbers of cultivable bacteria. However, the biodiversity of the microbiota associated with the gut is quite low, as only a few species dominate. *Vibrio* species are the main component of the gut microbiota in rearing conditions and are mainly derived from bacteria associated with the live prey (Fjellheim *et al.*, 2007; Skjermo and Vadstein, 1993; Verschuere *et al.*, 1997).

After mouth opening and the onset of exogenous feeding, it is relatively easy to manipulate the bacterial diversity in the gut by the use of bacteria bioencapsulated in prey as rotifers or *Artemia* metanauplii (Makridis *et al.*, 2000a). This phase is critical in many aspects for the successful rearing of marine fish larvae as pioneer species, i.e. bacterial populations that firstly colonize the gut, have a competitive advantage compared with bacteria that reach the gut at a later stage, when available adhesion sites have been reduced and environmental conditions are influenced by the bacterial populations already established (Hansen and Olafsen, 1999; Skjermo and Vadstein, 1999).

A main characteristic of the type of colonization in the gut of larvae is that species established during this period are mainly opportunistic, taking advantage of the input of nutrients in a "new" environment (larval gut). The establishment of excessive numbers of bacteria in weakened larvae may result in high mortalities, but mortalities in healthy larvae, not exposed to environmental stress and fed on high quality prey, are low and not dependent on the numbers of bacteria present in the gut (Makridis *et al.*, 2008; Salvesen *et al.*, 1999).

At the stage of weaning, the numbers of bacteria decrease and relatively few bacteria are measured in the fish gut per unit of gut weight compared with the earlier stages (Makridis *et al.*, 2008). The development of stomach and its acid barrier makes more complicated the direct influence of species established in the intestinal microbiota by addition of probiotics in the feed.

1.2. New strategies for bacterial infections control in fish larvae

The development of new strategies is based mostly on the study and knowledge of microbial diversity and ecology in marine fish larviculture systems (Figure 1.1). Strategies can be classified as based on:

i) Acting and modifying the microbiological communities of the system, including or not the introduction of live microorganisms.

ii) Acting on host-microbe interactions, which include the use of immunostimulants.

These new strategies can be applied to different developmental stages in fish larvae, from egg to juvenile stage (Figure 1.1). Each step of larval development is characterized by a different type of associated microbiota, which is introduced to the system by different ways: via seawater (in eggs and yolk-sac larvae) and via live prey (feeding larvae). Some strategies imply one single manipulation (e.g. addition of probiotics to the seawater), whereas others (e.g. bioencapsulation) involve the manipulation of the food chain. All these facts should be considered when selecting the strategy to control infections.

1.2.1. Acting on the microbiological communities of the system

1.2.1.1. Promoting beneficial bacteria in the system

- **Matured seawater**

A major change that occurs during transfer of seawater from the sea to a closed container is that seawater comes in contact with large solid surfaces in relation to its relatively small volume. These surfaces offer a new substrate for bacteria present in the seawater and thereby enhance the proliferation of opportunistic bacteria. Immediate use of this non-mature seawater has often led to mass mortalities during the rearing of marine fish larvae. The final aim of matured seawater is to reduce the percentage of opportunistic bacteria in the inflow seawater and thereby in the rearing system. A strategy has been developed, based on the retention of seawater in a large container where material with high surface to volume ratio has been placed under strong aeration for a long time period (optimally several weeks). Seawater is filtered through a membrane and the total numbers of bacteria are thus heavily reduced. The inflow rate of seawater and, thereby, the supply of nutrients is kept quite low inducing the proliferation of slow-growing, *K* strategist bacterial species.

This approach involves two steps. At first, a biofilm is formed on the surface of the material, at the interface with seawater, where bacteria are established at a high density, thereby favouring to *K*-strategists species. At the same time, a high bacterial load should be avoided in seawater in the rearing tanks. The second step of this strategy is the transfer of biofilm members to the seawater. A residence time of at least 24 h is considered necessary prior to use of matured seawater in the rearing of marine fish

larvae. A high residence time is accomplished by the use of large containers in combination with low inflow/outflow rates.

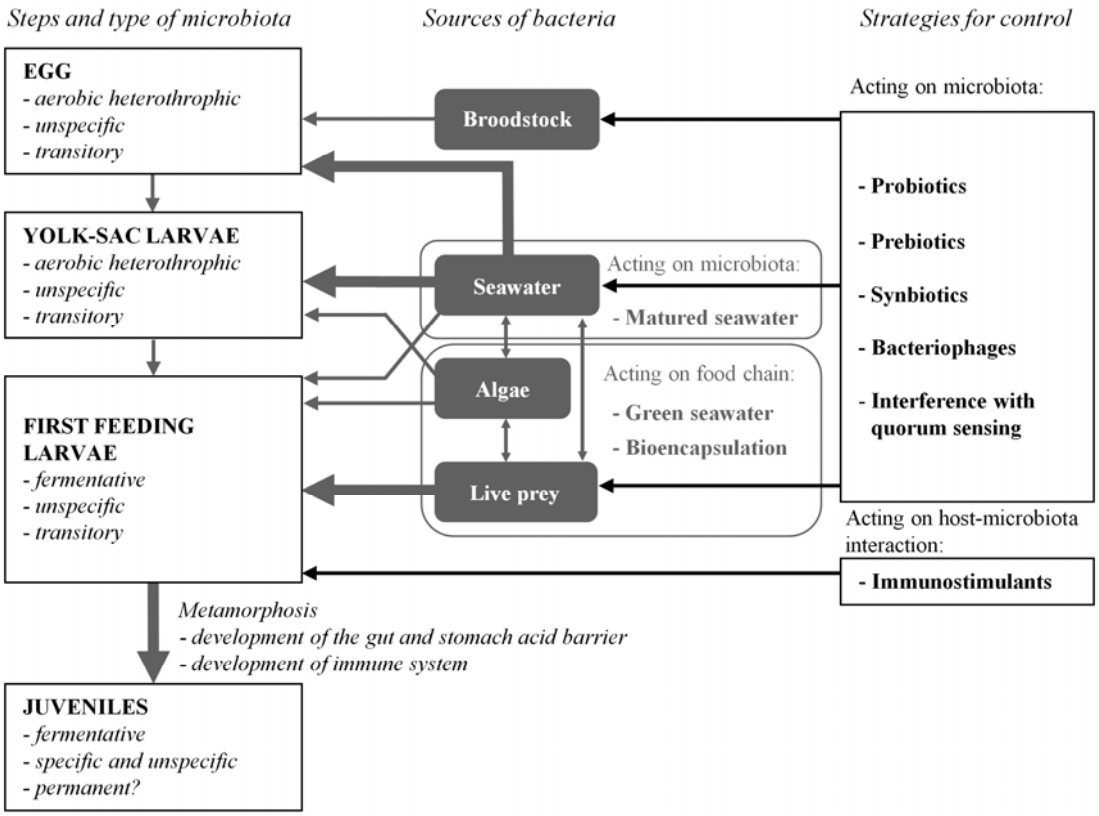

Figure 1.1. Application of new strategies for the control of bacterial infections in marine fish larval rearing.

A basic principle involved in the use of matured seawater is that, as non-opportunistic and less harmful bacteria species are at increased level in inflow seawater, they will prevent the proliferation of harmful opportunistic species. Slow-growing bacteria will thereby be present in high numbers during the first days after hatching and may have a competitive advantage over opportunistic bacteria, which proliferate at later stages of the rearing. So called "pioneer species" will colonize first various parts of the developing gut, and occupy adhesion sites.

Matured seawater has been successfully applied in several experiments with marine fish larvae, as in the case of incubation of Atlantic halibut yolk-sac larvae (Skjermo *et al.*, 1997) and with first feeding of turbot larvae (Salvesen *et al.*, 1999). It should be noted that in Recirculating Aquaculture Systems (RAS), there is an analogy

with matured seawater system, as in the biofilter there is a material with high surface to volume ratio, which offers the necessary support for the establishment of a biofilm with bacterial populations at a high density. Stable bacterial conditions are normally observed in RAS (Attramadal *et al.*, 2005).

- **Green seawater**

Use of microalgae has often resulted in successful rearing of marine fish larvae as determined by low mortalities, high growth and viable fry (Howell, 1979; Naas *et al.*, 1991; Reitan *et al.*, 1993). There are two different variants related to the application of microalgae in rearing of marine fish larvae. In the first case, microalgae are cultured in a large tank and, when optimal conditions are reached, the tank is stocked with fish eggs or larvae (green seawater technique). In this case, production of microalgae takes place in the same tank as the rearing of the larvae. In a more common approach, production of microalgae takes place in separate culture tanks; the microalgae are harvested regularly and added to the rearing tanks of the larvae (pseudogreen seawater technique).

Use of microalgae has several implications for different aspects of larval rearing. Marine fish larvae drink seawater to osmoregulate and ingest microalgae particles, which are accumulated in the gut. The phenomenon of "green gut" has been shown in the larval rearing of many marine fish species (Cahu *et al.*, 1998). Filling of the gut seems to have itself an effect on the stimulation of production of digestive enzymes in the larval gut (Hjelmeland *et al.*, 1988).

In the case that fish larvae are stocked at a low density, average residence time of live food organisms can be up to several days. Microalgae cells are available for the live food organisms present in the tank and may prevent the incidence of starved live food organisms (Makridis and Olsen, 1999).

Addition of microalgae changes the physicochemical parameters such as light, colour of tank seawater and concentration of ammonia and other harmful compounds, as well as to reduce light intensity in the seawater mass and the stress caused by direct light on the larvae.

Another aspect of addition of microalgae is that microalgae are normally produced in non-axenic cultures and high numbers of bacteria are present in the microalgae

cultures (Salvesen *et al.*, 2000). Microalgae are usually added together with their growth medium, so bacteria from microalgae cultures are added as well in the rearing tanks and may have an effect on the bacterial communities in the rearing tanks. Bacterial strains isolated from microalgae have shown *in vitro* antibacterial activity against fish pathogens (Makridis *et al.*, 2006). The antibacterial effect of microalgae can be involved in a strategy to reduce the bacterial load in live feed organisms, when rotifers or *Artemia* are incubated in microalgae cultures (Makridis *et al.*, 2006). It has been shown that an excessive bacterial content in live feed may overload the bacterial load in the rearing system and that larvae avoid live prey with high bacterial content (Pérez-Benavente and Gatesoupe, 1988).

- **Prebiotics**

Prebiotics are generally non-digestible diet compounds which cause a beneficial effect on the host by selecting growth or activating metabolism of one or a limited number of health-promoting bacteria (Gibson and Roberfroid, 1995). Research on application of prebiotics to aquaculture is still limited (Burr *et al.*, 2005; Gatesoupe, 2005; Gatlin *et al.*, 2006). The capacity of non-digestible carbohydrates to support the growth of probiotic bacteria has been proved *in vitro* (Rurangwa *et al.*, 2009) and *in vivo* trials have been conducted with dietary yeast culture or short chain fructo-oligosaccharides, which modified the intestinal microbial communities (Zhou *et al.*, 2009).

In fish larviculture, the prebiotics have a potential use in first feeding and during the weaning of larvae. Mahious *et al.* (2006), in assays with inulin and oligosaccharides in weaning of turbot (*Psetta maxima*) larvae, demonstrated a positive effect on growth and a predominance of *Bacillus* sp among bacterial isolates.

1.2.1.2. Adding beneficial bacteria

- **Probiotics**

Probiotics, defined by FAO/WHO (2001) as “live microorganisms which, when administered in adequate amounts, confer a health benefit on the host”, constitute a potential tool in the reduction of mortalities in the rearing of aquatic organisms

(Gatesoupe, 1999; Gram and Ringø, 2005; Kesarcodi-Watson *et al.*, 2008; Verschuere *et al.*, 2000; Vine *et al.*, 2006).

In rearing systems, where host and microorganisms share the same aquatic environment, the definition of probiotic was broadened by Verschuere *et al.* (2000) to include also the microorganisms which could exert a beneficial effect not colonizing the host (intestinal track, gills or skins) but being present in the seawater. This opens the concept to microorganisms acting on seawater quality or as biocontrol agents in seawater.

The different modes of action of probiotics include: production of inhibitory compounds (e.g. antibiotic, bacteriocins, organic acids and oxygen peroxide), competence for limiting nutrients (siderophores), enzymatic contribution to digestibility of feed, competence in adhesion to mucus, stimulation of immune system or improvement of seawater quality. As previously mentioned by Austin and Brunt (2009), there is still a lack of knowledge on the precise mode of action of most probiotics and, in some cases, the beneficial effect needs a combination of several mechanisms e.g. attachment and growth in mucus can be a previous requisite to exert antagonistic activity or immunostimulation on the host.

Most of the published work on probiotics in larviculture is applied research, with few evidences of the precise mode of action (Vine *et al.*, 2006). Moreover, many probiotic mechanisms have been studied *in vitro* which does not guarantee an *in vivo* effect (Gram *et al.*, 2001).

Hatching or early developmental stages, before first feeding, is an adequate period for the use of probiotics. At these stages, bacteria colonizing the eggs or larvae may prevent the further colonization of opportunistic bacteria originated from the diet (Carnevali *et al.*, 2004; Hansen and Olafsen, 1999; Olafsen, 2001). Another advantage is that, in contrast to adults, there is no acidic stomach barrier in most marine fish larvae and potential probiotics would not need to resist acidity (Rønnestad *et al.*, 2000; Tanaka *et al.*, 1996).

Probiotics in marine larviculture have been recently reviewed by Vine *et al.* (2006). The use of probiotics in fish larvae, summarized in Table 1.1, has been commonly focused on the use of commercially available or selected terrestrial lactic acid bacteria (Abelli *et al.*, 2009; Arndt and Wagner, 2007; Benetti *et al.*, 2008;

Carnevali *et al.*, 2004; Gatesoupe, 1991, 1994; Planas *et al.*, 2004; Plante *et al.*, 2007; Villamil *et al.*, 2003; Suzer *et al.*, 2008). Commercial yeasts have also been proposed as probiotics (Tovar-Ramírez *et al.*, 2002).

A better strategy, that avoids the introduction of non-native microorganisms to the system, is to select probiotic candidates among strains isolated from healthy fish (Meuer *et al.*, 2006; Olsson *et al.*, 1992; Ottesen and Olafsen, 2000; Westerdahl *et al.*, 1991) or hatchery facilities (Hjelm *et al.*, 2004a, b; Huys *et al.*, 2001). The efficacy of autochthonous probiotics is likely to be highest in the host species from which the bacteria were isolated from (Verschuere *et al.*, 2000).

Generally, selection is based on the antagonistic effect of the potential probiotic against pathogenic bacteria responsible of high mortalities in larvae, such as *Listonella anguillarum* (Olsson *et al.*, 1992; Planas *et al.*, 2006), *Vibrio* sp (Gatesoupe, 1994; Vine, 2004a, b) or *Aeromonas* sp (Ringø and Vadstein, 1998; Vine, 2004a, b), but *in vitro* growth and adhesion to mucus, immunostimulation or enhancement of enzymatic activity have been also used as criteria for probiotic selection (Gatesoupe, 1994; Picchetti *et al.*, 2009; Suzer *et al.*, 2008; Vine *et al.*, 2004a, b). It is important to notice that probiotics can be added directly to the seawater or introduced into the system previously bioencapsulated in live feed (Table 1.1). In some cases the final effect may depend on the selected way of delivery.

- **Synbiotics**

A synbiotic is defined as a combination of a probiotic with a prebiotic, usually a selective substrate, which ensures the growth and the permanence of the associated probiotic (Ziemer and Gibson, 1998). In aquaculture, the use of synbiotics has been proposed by Vine *et al.* (2006) but, at the present moment, only *in vitro* trials have been conducted to assay the potential of non-digestible carbohydrates and other prebiotics on the growth of probiotics (Rurandwa *et al.*, 2009).

Table 1.1. Probiotics and prebiotics evaluated to be used in marine fish larval rearing

Probiotic					
Gram-positive bacteria	Isolation / Source	Targeted fish species	Mechanism	Application	Reference
Arthrobacter sp	Renogen® (Novartis)	*Melanogrammus aeglefinus*	Modification of bacterial microbiota	Seawater	Plante *et al.* (2007)
Bacillus subtilis + *Bacillus licheniformis* + *Bacillus megaterium*	EcoPro™ (Eco Microbials)	*Rachycentron canadum*	Unknown	Rotifers and *Artemia*	Benetti *et al.* (2008)
Carnobacterium sp	Live food	*Psetta maxima*	Enhancement of dietary value of live food	Rotifers	Gatesoupe (1994)
Lactobacillus spp	Commercial probiotic mixture	*Onchorrynchus clarkia pleuriticus*	Unknown	*Artemia*	Arndt and Wagner (2007)
Lactobacillus spp	Biotexin and Protexin (Novartis)	*Sparus aurata*	Enhancement of enzymatic activity	Seawater, rotifers and *Artemia*	Suzer *et al.* (2008)
Lactobacillus delbrueckii	Gut of adult *Dicentrarchus labrax*	*Dicentrarchus labrax*	Stimulation of immune system	Rotifers and *Artemia*	Picchietti *et al.* (2009)
		Sparus aurata	Stimulation of immune system	Rotifers and *Artemia*	Abelli *et al.* (2009)
Lactobacillus fructivorans	Gut of *Sparus aurata*	*Dicentrarchus labrax* and *Sparus aurata*	Stimulation of immune system	Rotifers and *Artemia*	Abelli *et al.* (2009)
Lactobacillus fructivorans + *Lactobacillus plantarum*	Human faeces	*Sparus aurata*	Antagonism	Rotifers, *Artemia* and inert food	Carnevali *et al.* (2004)
Lactobacillus helveticus + *Streptococcus thermophilus*	Adjulact 1000 (Lallemand)	*Psetta maxima*	Enhancement of dietary value of live food	Rotifers	Gatesoupe (1991)
Lactobacillus plantarum	Bel Industries	*Psetta maxima*	Enhancement of dietary value of live food	Rotifers	Gatesoupe (1991)
Lactobacillus plantarum	Rotifers	*Psetta maxima*	Enhancement of dietary value of live food	Rotifers	Gatesoupe (1994)
Lactobacillus plantarum	*Gadus morhua*	*Hippoglossus hipoglossus* eggs	Enhancement of non-specific defence	Seawater	Ottesen and Olafsen (2000)
Lactobacillus plantarum	Human faeces	*Sparus aurata*	Antagonism	Rotifers, *Artemia* and inert food	Carnevali *et al.* (2004)
Paracoccus sp	Rotifers	*Sparus aurata*	Antagonism	Seawater	Makridis *et al.* (2005)
Pediococcus acidilactici	Bactocell	*Pollachius pollachius*	Antagonism	*Artemia*	Gatesoupe (2002)
Strain J84	Stomach of juvenile *Solea senagalensis*	*Solea senagalensis*	Antagonism	Seawater	Makridis *et al.* (2008)

Table 1.1. Continuation

Gram-negative bacteria	Isolation / Source	Targeted fish species	Mechanism	Application	Reference
Aeromonas sp	*Artemia*	*Sparus aurata*	Antagonism	Seawater	Makridis *et al.* (2005)
Cytophaga sp	Rotifers	*Sparus aurata*	Antagonism	Seawater	Makridis *et al.* (2005)
Phaeobacter sp	*Psetta maxima* rearing tank walls	*Psetta maxima*	Antagonism	Rotifers	Planas *et al.* (2006)
Roseobacter spp	*Psetta maxima* rearing tank walls	*Psetta maxima*	Antagonism	Seawater	Hjelm *et al.* (2004a, b)
Roseobacter sp	Rotifers	*Sparus aurata*	Antagonism	Seawater	Makridis *et al.* (2005)
Ruegeria sp	Rotifers	*Sparus aurata*	Antagonism	Seawater	Makridis *et al.* (2005)
Shewanella sp	Rotifers	*Sparus aurata*	Antagonism	Seawater	Makridis *et al.* (2005)
Shewanella sp	Hindgut of juvenile *Solea senegalensis*	*Solea senegalensis*	Antagonism	Seawater	Makridis *et al.* (2008)
Vibrio sp	*Psetta maxima*	*Psetta maxima*	Antagonism	Rotifers	Gatesoupe (1997)
Vibrio sp	Midgut of juvenile *Solea sene galensis*	*Solea senegalensis*	Antagonism	Seawater	Makridis *et al.* (2008)
Vibrio mediterranei	*Sparus aurata* larvae	*Psetta maxima*	Antagonism	Seawater	Huys *et al.* (2001)
Vibrio pelagius	*Psetta maxima* larvae	*Psetta maxima*	Antagonism	Seawater	Ringø and Vadstein (1998)
Vibrio salmonicida	Gut of *Salmo salar*	*Hippoglossus hipoglossus* eggs	Enhancement of non-specific defence	Seawater	Ottesen and Olafsen (2000)
Strain 4:44	*Psetta maxima* larvae	*Psetta maxima*	Colonization of larval gut	Seawater and rotifers	Makridis *et al.* (2000)
Strain PB52	*Psetta maxima* larvae	*Psetta maxima*	Colonization of larval gut	Seawater and rotifers	Makridis *et al.* (2000)
Yeasts	Isolation / Source	Targeted fish species	Mechanism	Application	Reference
Saccharomyces cerevisiae	Goteborg University Collection	*Dicentrarchus labrax*	Adhesion to the gut	Inert food	Tovar *et al.* (2002)
	Commercial product	*Oreochromis niloticus*	Colonization of larval gut	Inert food	Meurer *et al.* (2006)
Debaryomyces hansenii	Fish gut	*Dicentrarchus labrax*	Enhancement of enzymatic activity	Inert food	Tovar *et al.* (2002)
Prebiotic					
Identity	Isolation / Source	Targeted fish species	Mechanism	Application	Reference
Inulin and Oligosacharides	Raftiline ST, Raftilose P95 and Lactosucrose LS-55P.	Weaning *Psetta maxima*	Enhancement of *Bacillus* sp growth	Inert food	Mahious *et al.* (2006)

- **Biofilters**

In aquaculture, the concept of probiotics has been extended to bacteria which can exert their beneficial effect on the host by modification of the microbial community associated with fish or seawater, improving the quality of fish environment (Verschuere *et al.*, 2000). Strategies of seawater treatment with addition of beneficial bacteria would be also included.

Recirculating Aquaculture Systems (RAS) are the newest form of fish farming production system, more adapted to fulfil environmental constraints (e.g. generating low emissions or making coastal location unnecessary). RAS are typically an indoor system that allows for farmers to control environmental conditions all-year round, being considered better than conventional flow-through systems from an environmental (Neori *et al.*, 2007) and bio-security (Pruder, 2004) point of view. In the last years, RAS systems have been investigated for cultivation of different fish species larvae (Faulk and Holt, 2005; Martins *et al.*, 2009). Seawater quality of aquaculture facilities equipped with RAS is maintained by a diversity of microbial community associated with biofilters (Gross *et al.*, 2003; Michaud *et al.*, 2006; Sugita *et al.*, 2005). Recirculation of seawater tends to stabilize the microbial community and make it more persistent to perturbations (Attramadal *et al.*, 2005). Biofilters colonized with non-opportunistic bacteria have been previously used for production of matured seawater, which increased survival of Atlantic halibut yolk sac larvae (Skjermo *et al.*, 1997) and improved the growth of turbot larvae (Salvesen *et al.*, 1999) by improving microbial seawater quality.

Some probiotic bacteria, with antagonistic activity towards fish pathogens have also the capability to produce biofilms. Alpha proteobacteria belonging to the *Roseobacter* clade have demonstrated to produce tropodithietic acid (Brinkhoff *et al.*, 2004; Bruhn *et al.*, 2005; Porsby *et al.*, 2008), which inhibits different bacterial groups (Brinkhoff *et al.*, 2004) including some fish pathogens (Bruhn *et al.*, 2005; Porsby *et al.*, 2008; Ruíz-Ponte *et al.*, 1999). Production of this antibiotic is related to biofilm formation (Bruhn *et al.*, 2007). Therefore, biofilters could be inoculated and colonized by biofilm-forming probiotic bacteria, improving the permanence of the probiotics in the rearing system and protecting the larvae from opportunistic and potential pathogenic bacterial strains.

1.2.1.3. Inhibiting or inactivating detrimental bacteria in the system

- **Bacteriophages**

Bacteriophages are virus particles that infect bacteria. In nature, it has been suggested that bacteriophages are one of the main causes of mortalities in bacterial populations. The principle in the use of bacteriophages in aquaculture is their application for the control of bacterial diseases (Imbeault *et al.*, 2006; Park *et al.*, 2000). The advantage of such strategy is the avoidance of antibiotics use and other therapeutic agents. The drawback of this approach is that the ability of bacteriophages to reduce numbers of bacteria is characterized of high specificity. Production of virus particles specific for the pathogenic bacterial strains in question is therefore required at each time. In addition, resistance to bacteriophages is quickly developed after a relatively short period of time due to short generation time of bacteria.

The advantages of bacteriophage therapy against pathogenic bacteria are the following:

i) The high specificity results in protection of intestinal microbiota.

ii) The ability of propagation. Once susceptible bacteria are present in the fish, then few additions of bacteriophage may be sufficient for protection against pathogens (Nakai and Park, 2002).

There is a limited application, however, as mortalities in larviculture are seldom attributed to specific pathogens. In a specific hatchery however, it can occur that problems with mortalities in larvae rearing tanks are related to specific pathogens, so in such cases a tailor-made solution with bacteriophage therapy could be an alternative solution. Bacteriophages have shown encouraging results in the case of protection of shrimp larvae against *Vibrio harveyi* (Karunasagar *et al.*, 2007).

- **Interference with Quorum Sensing**

Quorum Sensing (QS) is a mechanism by which bacteria coordinate the expression of certain genes in response to their population density by producing, releasing and detecting small signal molecules, inducing a physiological response which

implies an ecological advantage (Defoirdt *et al.*, 2004). Gram negative bacteria use acylated homoserine lactones (AHLs) and/or autoinducer 2 (AI-2) as signal molecules (Fuqua *et al.*, 2001; Miller and Bassler, 2001; Whitehead *et al.*, 2001), whereas Gram positive bacteria use secreted peptides (Dunny and Leonard, 1997; Miller and Bassler, 2001).

Different bacterial phenotypes are controlled by a QS system, being the most important the expression of virulence factors and biofilm formation (De Kievit and Iglewski, 2000; Miller and Bassler, 2001; Whitehead *et al.*, 2001). The AI-2 mediated system in the well known fish pathogen *Vibrio harveyi* (Gómez-Gil *et al.*, 2004) is responsible for the virulence towards gnotobiotic *Artemia franciscana* (Defoirdt *et al.*, 2005), being also involved in the growth-retarding effect of this bacterium toward gnotobiotic *Brachionus plicatilis* (Tihn *et al.*, 2007). Although these two models of gnotobiotic *Artemia* and gnotobiotic rotifers are difficult to compare directly, it seems that *V. harveyi* QS systems might operate in a host-dependent way (Defoirdt *et al.*, 2005; Tinh *et al.*, 2007).

Disruption of QS was suggested as a new anti-infective strategy, as consequence of its importance in virulence development in pathogenic bacteria (Finch *et al.*, 1998). Several techniques have been developed for that purpose, such as inhibition of signal molecule biosynthesis, application of QS antagonists or agonistic analogues, chemical or enzymatic inactivation and biodegradation of QS molecules.

In aquaculture, disruption of QS might constitute an alternative approach for preventing or combating infections caused by pathogens that regulate virulence factor expression by a QS system (Defoirdt *et al.*, 2004). Halogenated furanones produced by the marine red algae *Delisea pulchra* (Manefield *et al.*, 1999) protected rotifers, *Artemia* and rainbow trout from the negative effect of vibrios when added in adequate concentrations (Defoirdt *et al.*, 2006; Rasch *et al.*, 2004; Tihn *et al.*, 2007). Furanones show structural similarity with AHL molecules and thus the mechanism of action would be by binding to LuxR proteins (Manefield *et al.*, 1999) present in pathogens as *V. harveyi* (Henke and Bassler, 2004).

The introduction of potential probiotic bacteria able to degrade QS molecules might be useful as biocontrol agents in aquaculture but the exploitation of these kind of bacteria remains a challenge for future research (Tihn *et al.*, 2008). It has been suggested that the positive effect of *Bacillus* spp strains used as probiotics in

aquaculture (Moriarty, 1998; Rengpipat *et al.*, 2003) might partly be due to inactivation of QS molecules, apart from the production of antagonistic substances (Dong *et al.*, 2000, 2004; Molina *et al.*, 2003). A direct immunomodulatory activity was produced in several mammalian systems by a QS molecule from *Pseudomonas aeruginosa* (Hooi *et al.*, 2004; Ritchie *et al.*, 2005; Thomas *et al.*, 2006).

The importance of QS *in situ* gene expression regulation is becoming more evident for pathogenic bacteria but hardly for putative probiotic bacteria. Some problems could arise, such as the development of resistances (Zhu *et al.*, 1998) or lack of specificity, as not all bacteria found to contain QS systems are pathogens. Therefore, there is a need to know more about the importance of QS molecules on their *in vivo* metabolism and their interaction with the non-pathogenic microbial community and with the host.

1.2.2. Acting on host-microbe interactions

- **Immunostimulants**

Fish are intimately in contact with a complex and dynamic microbial world, and a large fraction of these microbes cling to and colonize epithelial surfaces. In rare circumstances microbes cause disease, either directly, by damaging or traversing epithelial layers, or indirectly, by inducing tissue-damaging inflammatory responses. If microbial pathogens invade the host, innate and adaptive defence mechanisms are activated for preventing further spread of the infection (Gómez and Balcázar, 2008).

The fish immune system possesses two integral components:

i) The innate, natural or non-specific defence formed by series of cellular and humoral components.

ii) The adaptive, acquired or specific immune system characterized by the humoral immune response through the production of antibodies and by the cellular immune response mediated by T-lymphocytes.

However, it is believed that larvae do not have the ability to develop adaptive immunity during the early stages of development. In this regard, the innate immune system is probably the major defence against microbes (Vadstein, 1997). These

parameters include phagocytes, the complement system, various lectins, lytic enzymes, antibacterial peptides and proteinase inhibitors (Magnadottir *et al.*, 2005). A variety of substances could, at least theoretically, activate these innate parameters. These substances or immunostimulants have recently been defined as "a naturally occurring compound that modulates the immune system by increasing the host's resistance against diseases that in most circumstances are caused by pathogens" (Bricknell and Dalmo, 2005). In general, these are compounds of bacterial, fungal or plant origin, particularly composed of polysaccharides, which activate the pattern recognition receptors/proteins of the immune system resulting in a varied immune response.

Previous studies have demonstrated that administration of immunostimulants to fish is beneficial, providing improved protection against bacterial and, to a lesser extent, viral infections (Bricknell and Dalmo, 2005; Vadstein, 1997). Thus, the immunomodulation of larval fish has been proposed as a potential method for improving larval survival by increasing the innate responses until its adaptive immune response is sufficiently developed to generate an effective response to the pathogen.

Lipopolysaccharides (LPS) are one of the immunostimulants widely used in experimental fish larval aquaculture, as they can trigger various immune components. Sea bream (*Sparus aurata*) larvae when treated with LPS exhibited significantly high anti-protease activity, lysozyme activity and total globulin level, and also conferred protection against *Photobacterium damsela* (Hanif *et al.*, 2005). Similarly, Magnadottir *et al.* (2006) reported enhanced survival by immunising Atlantic cod (*Gadus morhua*) with LPS.

The range of potential immunostimulants for larval fish is quite limited and a short review can be found in Table 1.2. Although these studies have demonstrated some beneficial effects of immunostimulants in terms of survival and growth, the relative importance of the immune mechanisms involved remains unknown. It is also important to consider the specificity of immunostimulants, since the stimulation may be too intense, which may become detrimental or even lethal to the host. Moreover, the knowledge of the functions of different immunostimulators may be used to stimulate, more specifically, those parts of the immune system that may be more relevant in certain situations (Vadstein, 1997). The recent introduction of genomic and proteomic tools might be of special interest in this context.

Table 1.2. The immunostimulants used in fish larval aquaculture.

Immunostimulant	Species	Effect	Reference
Alginate	Turbot (*Psetta maxima*)	Survival improvement and growth increase	Conceicão *et al.* (2004)
Cod milt proteins	Atlantic cod (*Gadus morhua*)	Survival improvement	Pedersen *et al.* (2004)
β-1,3/β-1,6 glucans	Atlantic cod (*Gadus morhua*)	Survival improvement and growth increase	Skjermo *et al.* (2006)
	Common dentex (*Dentex dentex*)	Survival improvement and growth increase	Efthimiou (1996)
β-glucans and mannan-oligosaccharides	Chilean flounder (*Paralichthys adspersus*)	Survival improvement and growth increase	Piaget *et al.* (2007)
Lipopolysaccharide	Atlantic cod (*Gadus morhua*)	Survival improvement	Magnadottir *et al.* (2006)
	Sea bream (*Sparus aurata*)	Survival improvement	Hanif *et al.* (2005)

1.3. Strategies based on the food chain

Large scale use of probiotics in the rearing of marine fish larvae involves cultivation of bacteria in artificial medium, preparation of a stable form of these bacteria and development of a process for the transfer of probiotics to the larval gut either by direct addition to seawater in the rearing tanks or by bioencapsulation in live feed. The approach of addition of bacteria to the seawater is simpler but implicates that the added bacteria remain in the seawater column for some time to be filtered by rotifers or *Artemia* and thereby to be introduced in the larval gut. A far more efficient approach is the bioencapsulation of bacteria in live feed. This is the only possible route in the case of allochthonous bacteria of tellurian origin tested in larviculture, such as lactic acid bacteria and *Bacillus* strains (Gatesoupe, 1994). These bacteria are not normally encountered in marine systems, but when introduced in larvae may have positive effects for the larvae by excreting antimicrobial compounds or by other ways limiting the growth of harmful bacteria.

Bioencapsulation of bacteria is a relatively predictable process in the case of rotifers but more variable in the case of *Artemia* metanauplii, as the size of bacteria may prove decisive for the efficiency of the process (Makridis *et al.*, 2000). *Artemia* metanauplii have a filtering device which enables them to filter large bacterial cells or

aggregates of bacteria, although the efficiency of this process is not so high. Bioencapsulation has a dual purpose as, on one hand provides probiotic bacteria to the larvae and, on the other hand, replaces the microbiota originally found in the live feed, acting as control agent of opportunistic or pathogenic bacteria in live prey.

1.4. New molecular tools

The elucidation of the underlying mechanisms of action and the development of new strategies for microbial control requires the determination and characterization of the bacterial communities associated with the different compartments of the rearing system. If probiotic or pathogenic bacterial strains are introduced during *in vivo* challenges, the specific detection and quantification of introduced strains is also required.

Less than 1 % of total bacteria in natural seawater systems are cultivable (Hansen and Olafsen, 1999; Kjelleberg *et al.*, 1993) and thus the use of culture dependent methods leads to underestimate microbial diversity as well as to be time consuming. PCR based techniques such as Denaturing Gradient Gel Electrophoresis (DGGE) and real-time PCR, as well as non-PCR based techniques, such as Fluorescence *In Situ* Hybridization (FISH) have gained importance in microbial ecology studies (Amann *et al.*, 1990; Skovus *et al.*, 2007) and have been applied to different fish and shellfish species (Goarant and Merien, 2006; Griffiths *et al.*, 2001; Jensen *et al.*, 2004; Labreuche *et al.*, 2006; Prol *et al.*, 2009; Sandaa *et al.*, 2003; Schulze *et al.*, 2006). These methods typically rely on detection of microbial DNA to infer information on the microbial species within a sample and provide a unique perspective of the constituents of the microflora; however like culture based methods they also have limitations. These limitations range from technical problems such as obtaining representative genomic DNA and suitable primers, to conceptual problems such as defining and using meaningful taxonomic units of diversity (species) (Forney *et al.*, 2004).

DGGE is a reliable and rapid method to study variations of dominant bacteria and to characterize complex microbial populations (Muyzer *et al.*, 1993). The general principle of DGGE is the separation of fragments of the individual rRNA genes based on differences in chemical stability or melting temperature of the target genes. Seasonal variations of bacterial communities associated with live prey (McIntosh *et al.*, 2008; Rombaut *et al.*, 2001) and different fish (Griffiths *et al.*, 2001; Jensen *et al.*, 2004) and

shellfish (Bourne *et al.*, 2004; Payne *et al.*, 2006; Sandaa *et al.*, 2003) species have been successfully characterized by using DGGE. Recently, this fingerprint technique has been also used for detection and characterization of *Vibrio* strains in cod larvae (Reid *et al.*, 2009), to evaluate changes caused by different probiotic strains in rotifers microbial community composition (Qi *et al.*, 2009a) and applied to the study of immunostimulants in aquaculture (Liu *et al.*, 2008).

Real-time PCR is a specific and sensitive method for quantification of bacteria (Klein, 2002). This technique is based on the use of specific primers and fluorescent molecules which permit to measure the quantity of a specific amplicon at each PCR cycle. Two main chemistries have been developed: SYBR Green, which is based on the use of a double stranded DNA dye, and TAQMAN, based on specific oligonucleotide probes which have a fluorescent dye and a quencher emitting fluorescence only when new copies of the target gene are created. In aquaculture, different real-time PCR protocols have been designed, focusing on detection and quantification of pathogenic vibrios in crustaceans (Goarant and Merien, 2006), molluscs (Labreuche *et al.*, 2006) or fish (Prol *et al.*, 2009). Recently, real-time PCR has demonstrated to be a reliable technique for specific detection and quantification of introduced pathogenic and probiotic strains in first feeding turbot larvae during a challenge trial (Prol *et al.*, 2009).

FISH with rRNA target probes has been developed for the *in situ* identification of single microbial cells (Amann *et al.*, 1990). This method is based on the hybridization of synthetic oligonucleotide probes to specific regions within the bacterial ribosome and does not require cultivation. Due to its speed and sensitivity, this technique is considered a powerful tool for phylogenetic, ecological, diagnostic and environmental studies in microbiology (Bottari *et al.*, 2006). In aquaculture, this technique has been used to determine bacterial composition in a Pacific white shrimp hatchery (García and Olmos, 2007) and recently, a probiotic photosynthetic bacteria mixture used in aquaculture was characterized by applying a group of species-specific probes (Qi *et al.*, 2009b) but it was not already applied to fish larval rearing.

In studies of host-microorganisms interactions, gene profiling can be carried out by microarray technology which allows quantification of the transcriptional status of thousands of genes simultaneously (Douglas, 2006) and could help to elucidate global patterns of gene expression (Arcand *et al.*, 2004). For the construction of a cDNA microarray, cDNA fragments or EST (Expressed Sequenced Tags) sequences for the fish species of interest must be prepared from cDNA library or PCR-amplified products.

Transcriptome sequences of many fish species are now available in the National Centre for Biotechnology Information (NCBI) GenBank database, and these collected EST sequences can be used to synthesize oligonucleotides and can be spotted onto array chips to produce cDNA microarrays. Microarray analyses have been used to search for immune-related genes that are expressed following DNA vaccination or infection by fish pathogens (Byon *et al.*, 2005; Kurobe *et al.*, 2005) and to study environmental influences on gene expression of fish larvae (Williams *et al.*, 2003), fry (Koskinen *et al.*, 2004) and adults (Lam *et al.*, 2006). Hossain *et al.* (2006) found, by using microarray technology, that the differences between virulence determinant functions of different pathogens can be reflected by specific host gene expression patterns, suggesting their adaptative survival strategies inside de host.

1.5. Conclusion and perspectives

The intensive rearing of marine fish larvae is highly susceptible to bacterial infections. Environmental and health concerns have resulted in a progressive reduction of the use of chemotherapeutics and in the search of alternatives, which should be based on considering bacteria as active part of the aquaculture ecosystem. The selection of the appropriate strategy for microbial control would rely on a deep understanding of the microbial ecology in fish larvae, live food and rearing systems, of the origin of bacterial infections (specific pathogens or unspecific opportunistic bacteria) and of the host-bacteria interactions.

Tailored solutions should be developed for each case. In this sense, matured seawater, green seawater, biofilters or immunostimulants are more unspecific, than prebiotics, probiotics or synbiotics. All of them are directed to prevent or to enhance particular bacterial groups and would be suitable when larval mortality is attributed to opportunistic bacteria. The interference with QS would be applied to prevent specifically bacterial infections due to pathogens which use QS regulated virulence expression. The use of bacteriophages, highly specific, would be a strategy to be used only when the aetiology of infections has been proved to correspond to a specific pathogen.

The way how the host reacts to the presence of a particular microbiota should be a subject of further research. Long-term effects need also to be studied. The development of new molecular tools will certainly improve highly the knowledge on microbial

ecology and host-microbe interactions and on the underlying *in vivo* mechanisms of action of the different strategies available but not sufficiently understood in many cases.

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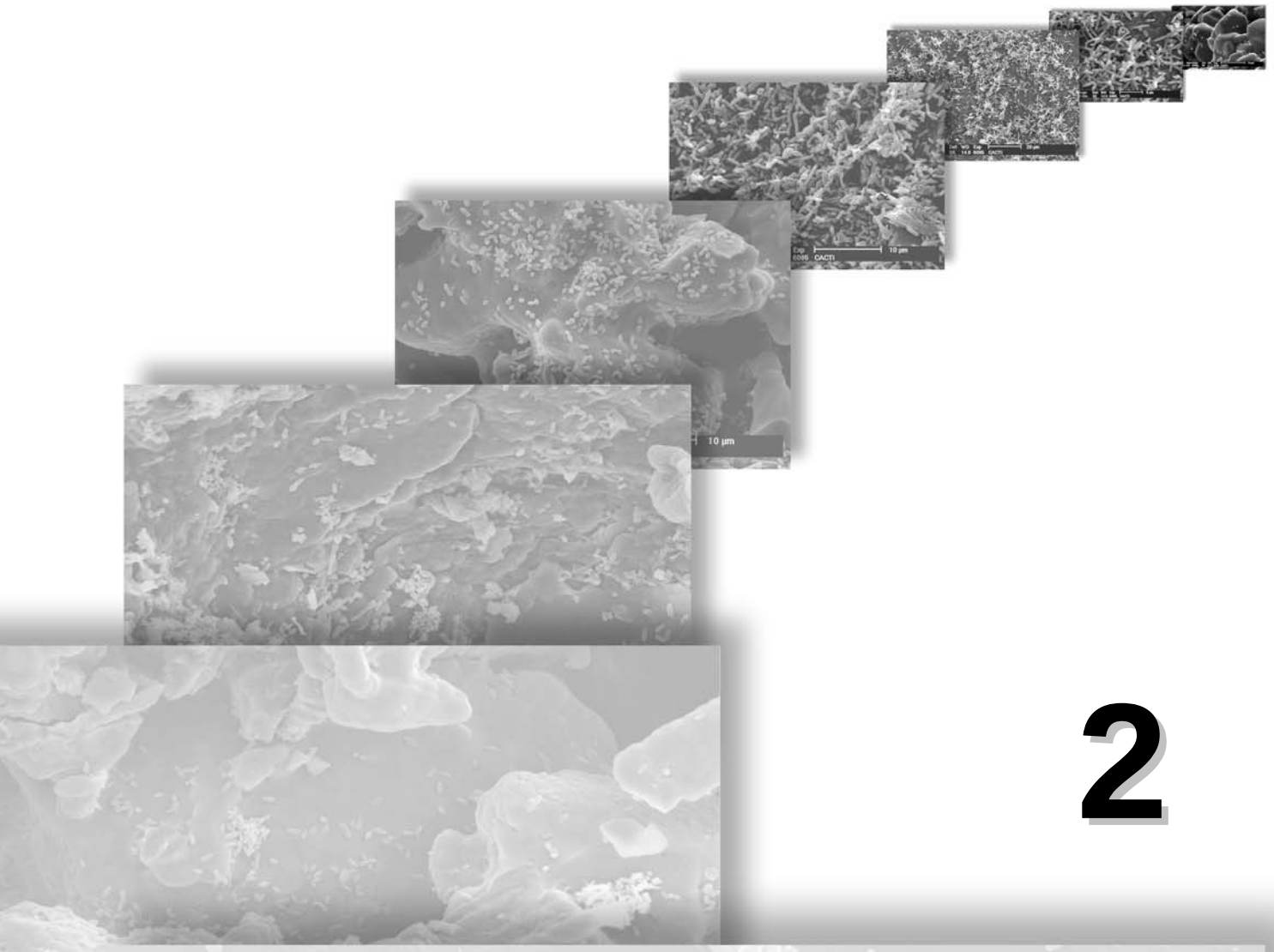
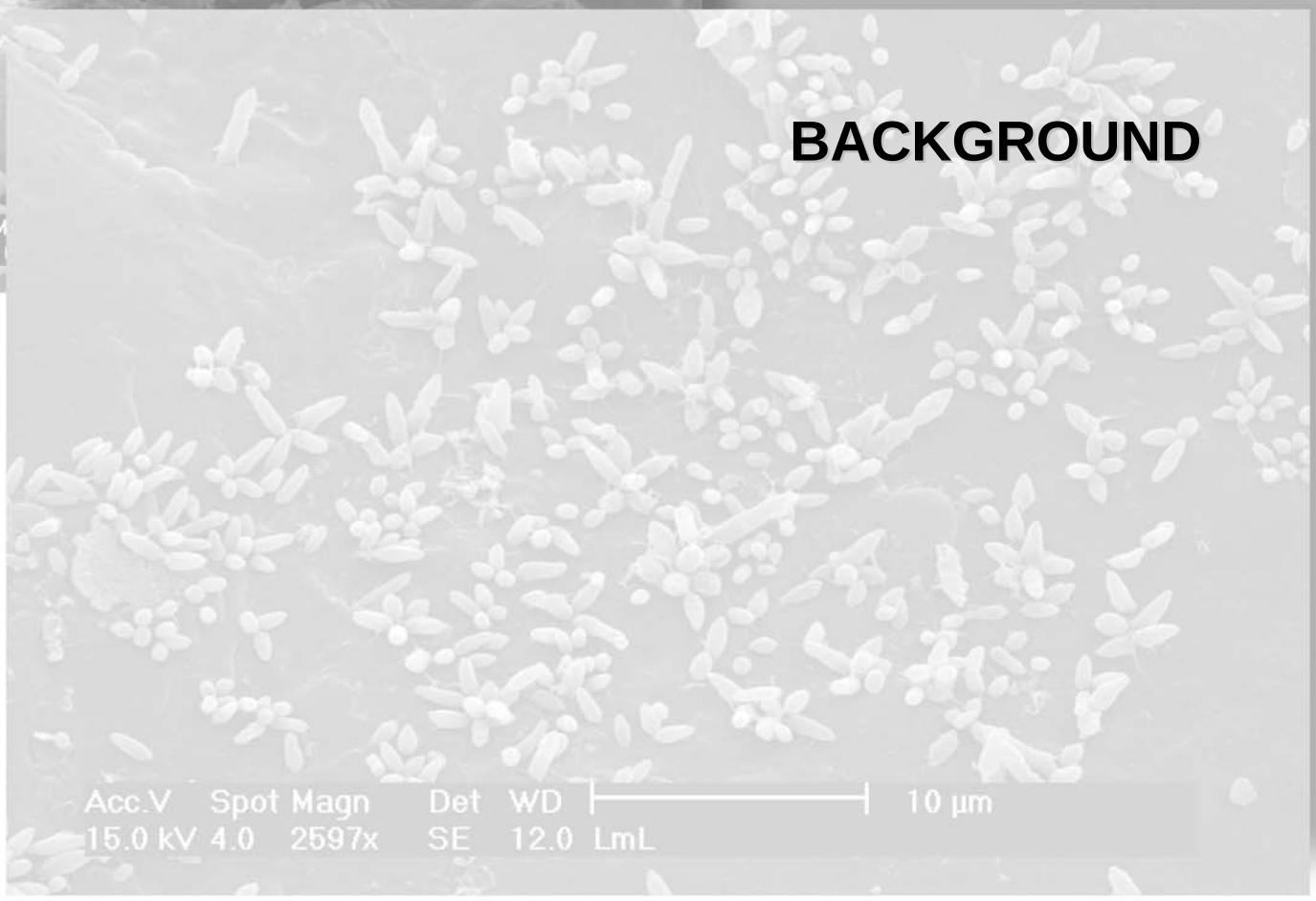


2

BACKGROUND



2. BACKGROUND

Phaeobacter 27-4 as probiotic against *Vibrionaceae* infections in turbot larvae

Generally, important losses in turbot farms are consequence of larval mortality during the first feeding on rotifers and *Artemia* (Ringø and Birbeck, 1999). The mortality is typically caused by opportunistic pathogenic bacteria, such as *Vibrionaceae*. Among them, some species such as *Listonella anguillarum* or *Vibrio splendidus* have been found to enter in the rearing systems via the live food (Thomson *et al.*, 2005; Verdonck *et al.*, 1997), causing mortality in fish larvae (Reid *et al.*, 2009; Sandlund and Bergh, 2008; Thomson *et al.*, 2005). This fact has been demonstrated by the higher survival rate observed in larvae fed on axenic rotifers (Munro *et al.*, 1995).

The entry route of *Vibrionaceae* bacteria into fish (via live prey or via seawater) (Grisez *et al.*, 1996; Muroga *et al.*, 1990; Planas *et al.*, 2005) together with their chemotaxis and capability of adhesion to mucosal surfaces (skin, gill, intestine) (Balebona *et al.*, 1995, Bordas *et al.* 1998) would determine different infection patterns, as well as the infection of different organs (Grisez *et al.*, 1996; Olsson *et al.*, 1996; Villamil *et al.*, 2003).

Strains *L. anguillarum* 90-11-287 and *V. splendidus* DMC-1 have demonstrated to be pathogenic for turbot larvae though the infection patterns for both species seem to be different. *V. splendidus* has been detected and isolated from the gut of diseased larvae (Reid *et al.*, 2009; Thomson *et al.*, 2005) whereas *L. anguillarum* has been found associated with the epidermis of experimentally infected turbot larvae (Planas *et al.*, 2005).

Several studies demonstrated that the intestinal microbiota in first feeding turbot larvae is more dependent on the bacterial community of live prey than on the microbiota present in the seawater (Blanch *et al.*, 1997; Munro *et al.*, 1993; Reitan *et al.*, 1998). Nevertheless, it has been reported that the epidermis of turbot larvae fed on rotifers infected with *L. anguillarum* was severely affected, and that the pathogen was detected in ingested rotifers but not in the intestinal epithelium (Planas *et al.*, 2005). The transference of *Vibrionaceae* pathogens from live prey to fish larvae can occur by direct

infection, by ingestion of the prey, and/or indirectly, by release of pathogens from prey to seawater. Additionally, *Vibrionaceae* bacteria may proliferate in the faeces of the larvae, which are composed by digested and non-digested live prey (Olsson *et al.*, 1998). Consequently, the load of *Vibrionaceae* bacteria in the surrounding seawater would increase and the pathogen might penetrate into the larvae through skin and gills.

Thus, the way pathogens and fish larvae come in contact could determine the colonization of the intestine and/or the adhesion to external epithelia (gill, skin) of larvae by pathogens. Therefore, as a first step to develop effective prevention strategies (e.g. probiotics), it is essential to understand the ecology of *Vibrionaceae* bacteria in live prey cultures (e.g. rotifers) and comprehend the mode fish larvae get infected.

Probiotics have arisen as a new strategy to improve the viability of fish larvae, avoiding the use of antibiotics and disinfectants (Gatesoupe, 1999; Irianto and Austin, 2002; Planas *et al.*, 2006; Verschuere *et al.*, 2000; Vine *et al.*, 2006). As mentioned in the previous chapter, besides the appearance of bacterial resistance to antibiotics (Cabello, 2006), the use of both antibiotics and disinfectants can result in the loss of a stable microbial population (*K* strategists), leading to a rapid colonization of the rearing systems by opportunistic bacteria (*r* strategists), such as *Vibrionaceae* (Skjermo and Vadstein, 1993).

In the searching for probiotic bacteria on marine fish larvae, different selection strategies have been applied (Gram and Ringø, 2005). *In vitro* inhibitory effect towards pathogens known to occur in larviculture has been frequently used to select potential probiotic candidates (Hjelm *et al.*, 2004a, b; Jorquera *et al.*, 1999; Westerdhal *et al.*, 1991). In the last years, potential probiotic strains selected according to their antagonistic activity include Gram positive bacteria as *Lactobacillus brevis* (Villamil *et al.*, 2003), *L. fructivorans*, *L. plantarum* (Carnevali *et al.*, 2004), *Paracoccus* sp (Makridis *et al.*, 2005) or *Pediococcus acidilactici* (Gatesoupe, 2002) and Gram negative bacteria of the genera *Aeromonas, Cytophaga* (Makridis *et al.*, 2005), *Marinomonas* (Fjellheim *et al.*, 2007), *Phaeobacter* (Hjelm *et al.*, 2004a, b; Makridis *et al.*, 2005; Planas *et al.*, 2006), *Pseudoalteromonas* (Fjellheim *et al.*, 2007), *Ruegeria* (Gram *et al.*, 2009; Makridis *et al.*, 2005; Porsby *et al.*, 2008), *Shewanella* (Fjellheim *et al.*, 2007; Makridis *et al.*, 2005, 2008) or *Vibrio* (Gatesoupe, 1997; Hjelm *et al.*, 2004b; Huys *et al.*, 2001; Makridis *et al.*, 2008; Ringø and Vadstein, 1998). Some of these isolated strains demonstrated to antagonize *Vibrionaceae* bacteria, such as *L. anguillarum* (Gram *et al.*, 2009; Hjelm *et al.*, 2004a, b; Makridis *et al.*, 2008; Olsson *et*

al., 1992; Planas *et al.*, 2006; Porsby *et al.*, 2008), *Photobacterium damselae* subsp *piscicida* (Makridis *et al.*, 2008), *Vibrio alginolyticus* (Vine *et al.*, 2004a, b) or *Vibrio splendidus* (Hjelm *et al.*, 2004a, b).

Members of the *Roseobacter* clade (α-Proteobacteria) (Brinkhoff *et al.*, 2008), a group globally distributed across diverse marine environments (Buchan *et al.*, 2005; Wagner-Döbler and Biebl, 2006), include several strains with demonstrated antagonistic activity against different taxonomic bacterial groups, by the production of secondary metabolites (Brinkhoff *et al.*, 2004; Grossart *et al.*, 2004; Hjelm *et al.*, 2004a, b; Porsby *et al.*, 2008). This competitive advantage of Roseobacters has led to study their application as probiotics in aquaculture (Hjelm *et al.*, 2004a, b; Planas *et al.*, 2006) or antifouling agents in marine systems (Egan *et al.*, 2008; Rao *et al.*, 2006).

Roseobacter clade strains of the genera *Phaeobacter* (formerly *Roseobacter*, Martens *et al.*, 2006) and *Ruegeria*, have the capability to produce tropodithietic acid and/or thiotropocin (Brinkhoff *et al.*, 2004; Bruhn *et al.*, 2005; Porsby *et al.*, 2008). It has been shown that these compounds are responsible of the inhibition of several bacteria (Brinkhoff *et al.*, 2004), including some fish pathogenic *Vibrionaceae* (Bruhn *et al.*, 2005; Hjelm *et al.*, 2004a, b; Porsby *et al.*, 2008). The addition of bacterial cultures or cell extracts of the antagonistic strains *Phaeobacter gallaeciensis*, *Phaeobacter* spp (Balcázar *et al.*, 2007; Planas *et al.*, 2006; Ruíz-Ponte *et al.*, 1999) or *Ruegeria* sp (Makridis *et al.*, 2005) into the rearing tanks have shown to produce a beneficial effect on larvae of fish (Makridis *et al.*, 2005; Planas *et al.*, 2006) and shellfish (Balcázar *et al.*, 2007; Ruíz-Ponte *et al.*, 1999).

Phaeobacter strain 27-4 was isolated from a turbot farm in Galicia (Stolt Sea Farm, Merexo, North Western coast, Spain) and identified, by sequencing of 16S rDNA, as 99 % similar to *Phaeobacter gallaeciensis* (Hjelm *et al.*, 2004b). This strain antagonizes different fish pathogens of *Vibrionaceae* family, such as *Listonella anguillarum* and *Vibrio splendidus* (Hjelm *et al.*, 2004a, b), as well as, a pathogenic *Tenacibaculum* sp strain, belonging to *Cytophaga-Flexibacter-Bacteroides* group, isolated from diseased turbot (Pintado *et al.*, in press).

Phaeobacter 27-4 is innocuous for turbot larvae and for the rotifers used as the first live prey (Pérez-Lorenzo, 2006; Pintado *et al.*, in press; Planas *et al.*, 2006), as well as for *Isochrysis galbana* (unpublished data), a microalgae commonly used for enrichment of live prey. This *Phaeobacter* strain, when delivered bioencapsulated in

rotifers to turbot larvae, increased the survival of larvae experimentally infected with *Listonella anguillarum* (Planas *et al.*, 2006). However, 27-4 did not colonize larval gut or rotifers being its permanence in the seawater of rearing tanks limited (Pérez-Lorenzo, 2006; Pintado *et al.*, in press; Planas *et al.*, 2006). This limited residence time implied the need of repeated additions of the probiont to larval cultures, which could make difficult the use at industrial scale.

Phaeobacter 27-4 does not form part of the normal microbiota of larvae. This fact might explain its inability to colonize turbot larvae (Hjelm *et al.*, 2004a). In a year-round study carried out in two turbot rearing plants in Galicia (North West Spain), it was found that *Phaeobacter* genus was predominant between the isolates showing antagonism against *Vibrionaceae* fish pathogens. Most antagonistic Roseobacters were isolated from the walls of rearing tanks and only low numbers were detected in seawater of tanks and larvae, which were fed on rotifers and *Artemia* (Hjelm *et al.*, 2004a).

Marine *Roseobacter* clade members are able to rapidly colonize a variety of inorganic and organic marine surfaces, including marine algae and dinoflagellates (Dang and Lovell, 2002; Mayali *et al.*, 2008), and are considered the primary surface colonizers (Dang *et al.*, 2008). The antagonistic potential of Roseobacters seems to be associated with their capability to grow on surfaces (Slightom and Buchan, 2009) and particle-associated members show a higher antimicrobial activity than free-living ones (Long and Azam, 2001). On the other hand, Quorum Sensing (QS) molecules have been related to surface colonization and production of secondary metabolites by different microorganisms (Davies *et al.*, 1998; Egan *et al.*, 2008; Labatte *et al.*, 2007). Recent studies suggest that Roseobacters might be the dominant producers of the QS molecules acyl homoserine lactones (AHL) in the marine environment (Cicirelli *et al.*, 2008).

Phaeobacter 27-4 has the capability to produce AHL molecules (Bruhn *et al.*, 2005, 2007), attach to inert surfaces and subsequently form a biofilm composed by rosette-shaped microcolonies (Bruhn *et al.*, 2006). The production of the antibacterial compound tropodithietic acid by *Phaeobacter* 27-4 has been associated with the production of a brownish pigment and with the biofilm formation in air-liquid interface (Bruhn *et al.*, 2005, 2007). It has been demonstrated that culture conditions influence the production of AHL molecules, the formation of rosettes and, in consequence of a biofilm, as well as, the production of the antagonistic compound and the pigment. All these features are favoured by the culture of the probiont under stagnant conditions (Bruhn *et al.*, 2005, 2006, 2007).

All these results suggest that biofilm forming bacteria with antagonistic activity, such as *Phaeobacter* 27-4, could be used as probiotic by means of its immobilization in biofilters and would reduce the level of pathogenic or total bacteria in seawater, live prey or fish larvae, as previously demonstrated by the addition of probiotics to the seawater of fish rearing tanks (Kennedy *et al.*, 1998; Makridis *et al.*, 2001). Those biofilters would enhance the permanence of the probiotic bacteria in the rearing systems, both in open or recirculating systems, protecting fish larvae against opportunistic and potential pathogenic bacterial strains such as *Vibrionaceae*.

2.1. References

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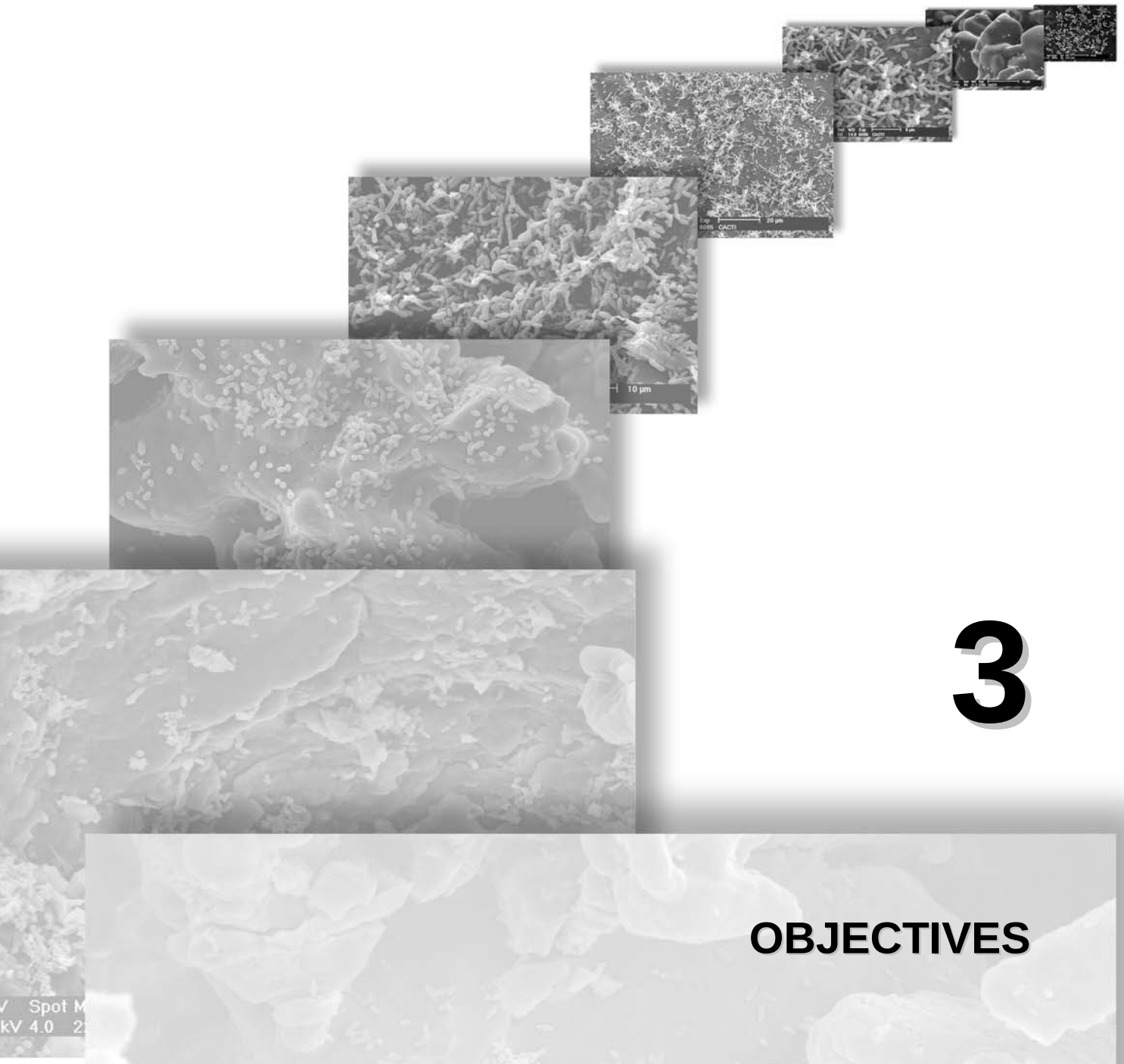


3

OBJECTIVES

3. OBJECTIVES

The main objective of the present Ph.D. Thesis was to study the immobilization of the antagonistic and biofilm forming probiotic bacterium *Phaeobacter* 27-4 in biofilters and to demonstrate its effectiveness for the control of *Vibrionaceae* infections in marine fish larval rearing.

Biofilters colonized by biofilm forming bacteria with antagonistic activity could exert a probiotic effect in fish larvae by reducing the level of pathogenic or opportunistic bacteria in seawater, live food (rotifer, *Artemia*) or fish larvae. This new application procedure would guarantee the permanence of the probiotic in the rearing system and protect turbot (*Psetta maxima*) larvae against pathogenic *Vibrionaceae* bacteria infections and increase larval survival at the beginning of exogenous feeding.

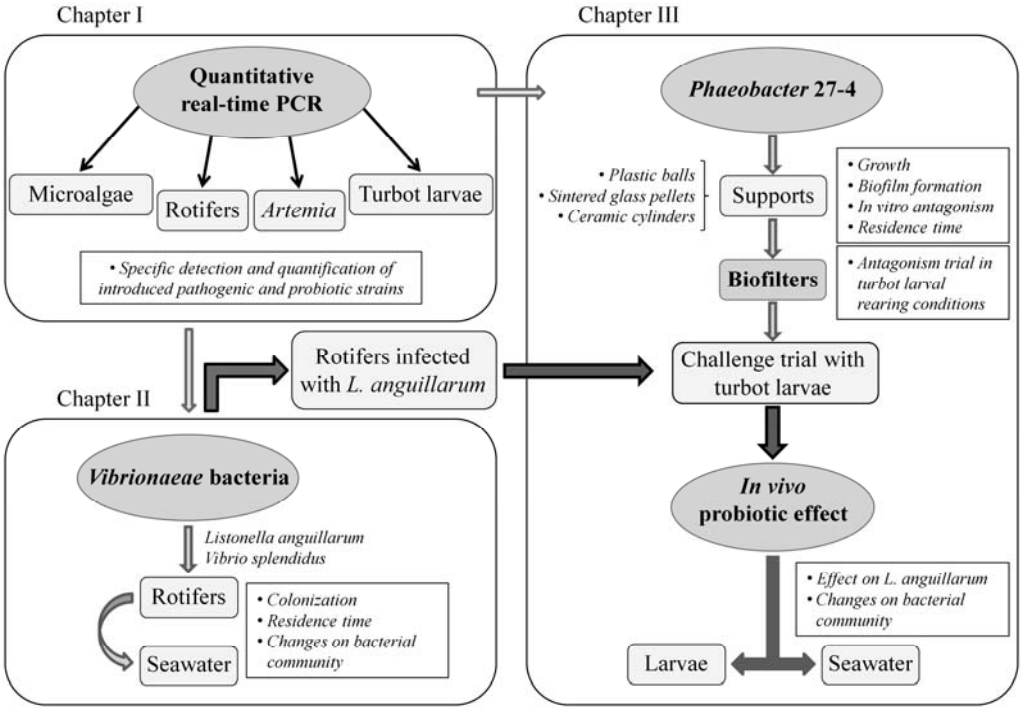


Figure 3.1. Description and interactions of the specific objectives developed in this Ph.D. Thesis. Chapter I: Real-time PCR detection and quantification of the fish probiotic *Phaeobacter* strain 27-4 and two fish pathogenic *Vibrionaceae* (*Listonella anguillarum* 90-11-287 and *Vibrio splendidus* DMC-1) in microalgae, rotifer, *Artemia* and first feeding turbot (*Psetta maxima*) larvae; Chapter II: Different colonization and residence time of *L. anguillarum* and *V. splendidus* in the rotifer *Brachionus plicatilis* determined by real-time PCR and DGGE; Chapter III: Application of biofilters with the probiotic strain *Phaeobacter* 27-4 against *Vibrionaceae* infections in the rearing of turbot (*Psetta maxima*) larvae.

To accomplish with this objective, a real-time PCR technique was developed to specifically detect and quantify both probiotic and pathogenic strains in larval rearing systems (Chapter I). As rotifer is the usual way of entry for potential pathogenic *Vibrionaceae* into fish larvae, the colonization and residence time of two pathogenic *Vibrionaceae* strains (*L. anguillarum* 90-11-287 and *V. splendidus* DMC-1) in rotifers were studied (Chapter II). The growth of the probiont *Phaeobacter* 27-4 on different supports, its residence time and *in vitro* antagonistic activity, and the *in vivo* probiotic effect of the probiotic immobilized in biofilters on turbot larvae challenged with a fish pathogen were evaluated (Chapter III).

The specific objectives addressed in this Ph.D. Thesis were distributed in three chapters as follows (Figure 3.1):

1. To develop and optimize a SYBR Green real-time PCR protocol for the specific detection and quantification of the probiont *Phaeobacter* 27-4 and the turbot larvae pathogens *L. anguillarum* 90-11-287 and *V. splendidus* DMC-1 in the organisms involved in turbot larval rearing: microalgae (*Isochrysis galbana*) used for the enrichment in fatty acids of the live food, rotifers (*Brachionus plicatilis*) and *Artemia* used as live prey, and turbot (*Psetta maxima*) larvae (Chapter I).

2. To study the colonization and residence time of the fish pathogens *Listonella anguillarum* 90-11-287 and *Vibrio splendidus* DMC-1 in the rotifer *Brachionus plicatilis* (Chapter II), including:

    2.1. The evaluation of colonization and the monitoring of both pathogens residence time in rotifers once transferred to turbot larvae rearing tanks and maintained under habitual rearing conditions by application of real-time PCR.

    2.2. The analysis of changes in bacterial populations of rotifers and seawater, as consequence of the introduction of fish pathogens, by application of a DGGE technique.

3. To study the application of *Phaeobacter* 27-4 in biofilters (Chapter III) against *Vibrionaceae* infections in turbot larval rearing, including:

    3.1. The selection of a support and *Phaeobacter* 27-4 culture conditions based on:

3.1.1. Growth and biofilm formation of *Phaeobacter* 27-4 on different biofilter supports used in aquaculture (plastic balls, sintered glass pellets and ceramic cylinders) and under different culture conditions (soaked and submerged with or without agitation).

3.1.2. *In vitro* antagonistic activity of *Phaeobacter* 27-4 biofilters against *L. anguillarum* 90-11-287 and *V. splendidus* DMC-1 by performance of inactivation and growth inhibition experiments in microcosms.

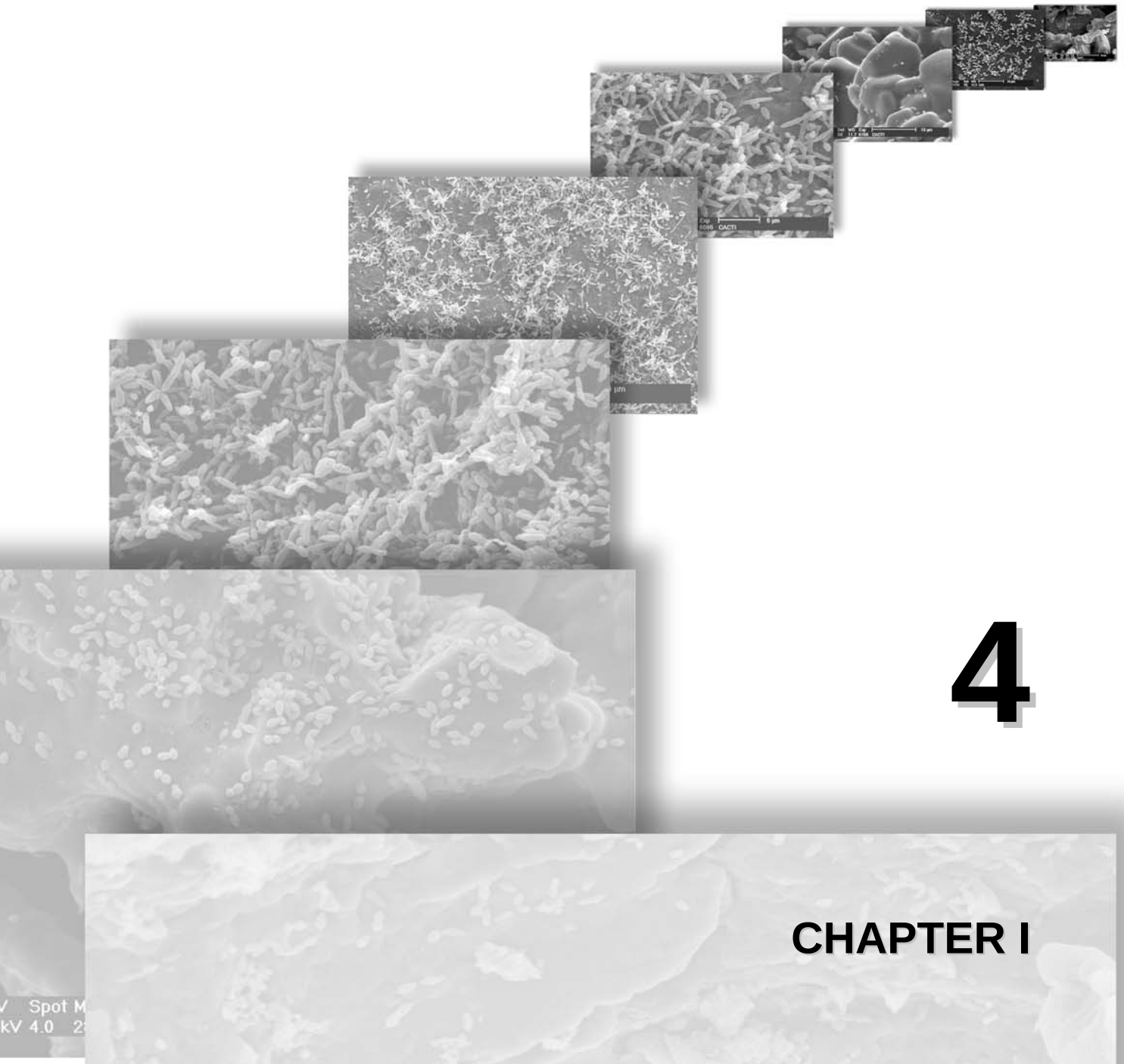
3.1.3. Residence time of the probiotic bacteria in biofilters prepared with different supports when maintained under turbot larval rearing conditions.

3.2. With the selected support and *Phaeobacter* 27-4 culture conditions, the demonstration of:

3.2.1. The effect of *Phaeobacter* 27-4 biofilters against *L. anguillarum* 90-11-287 in green seawater under turbot larval rearing conditions and different nutrients levels.

3.2.2. The *in vivo* probiotic effect of *Phaeobacter* 27-4 biofilters, on turbot larvae challenged with *L. anguillarum* 90-11-287 introduced via rotifers.

3.2.3. The relationship between turbot larvae survival and the presence of *Phaeobacter* 27-4 or the modification of bacterial microbiota in larvae and/or seawater.



4

CHAPTER I

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4. CHAPTER I

Real-time PCR detection and quantification of the fish probiotic *Phaeobacter* strain 27-4 and two fish pathogenic *Vibrionaceae* in microalgae, rotifer, *Artemia* and first feeding turbot (*Psetta maxima*) larvae

4.1. Introduction

Turbot larval mortality during the first feeding on rotifers and *Artemia* is the cause of important losses in turbot farms (Ringø and Birbeck, 1999). This limits the supply of juvenile fish and, in consequence, the expansion of turbot production in aquaculture (Riaza and Hall, 1993). The mortality is typically caused by opportunistic pathogenic bacteria that enter the rearing systems via the live food (Chair *et al.*, 1994; Grisez *et al.*, 1996). Thus, larvae fed axenic rotifers have a higher survival rate (Munro *et al.*, 1995).

Vibriosis is one of the main causes of mortality in turbot (Grisez *et al.*, 1996; Thomson *et al.*, 2005; Toranzo *et al.*, 1993). *Listonella anguillarum* and *Vibrio splendidus* are part of the normal microbiota in turbot farms (Thomson *et al.*, 2005; Toranzo *et al.*, 1993) and both species have been associated with infectious episodes (Angulo *et al.*, 1994; Toranzo *et al.*, 1993).

The mortalities may be reduced or eliminated by antibiotics, however, the concerns raised with respect to antibiotic resistance in bacteria has led to searches for alternative disease control measures. One such alternative is the use of probiotics (Vine *et al.*, 2006), defined by FAO/WHO as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host". Probiotic strains may improve seawater quality (Dalmin *et al.*, 2001), reduce the level of pathogenic bacteria (Kennedy *et al.* 1998), reduce the total bacterial load (Makridis *et al.*, 2001), or increase the growth rate of live food (Planas *et al.*, 2004). All of these features will increase the survival of fish (Gram *et al.*, 1999; Hjelm *et al.*, 2004a; Kennedy *et al.*, 1998; Planas *et al.*, 2006) and shellfish (Ruíz-Ponte *et al.*, 1999).

Several species of the *Roseobacter* clade (α-Proteobacteria) antagonize fish pathogenic and other bacteria (Brinkhoff *et al.*, 2004, 2008; Hjelm *et al.*, 2004a, b; Ruíz-Ponte *et al.*, 1999) and some, such as *Phaeobacter* strains, have an *in vivo* probiotic effect in challenge trials with scallop (Ruíz-Ponte *et al.*, 1999) or turbot larvae (Hjelm *et al.*, 2004a; Planas *et al.*, 2006). *Phaeobacter* strain 27-4 antagonizes *L. anguillarum* and *V. splendidus* (Hjelm *et al.*, 2004a, b), due to the production of tropodithietic acid and/or thiotropocin (Bruhn *et al.*, 2005). Feeding *L. anguillarum* challenged larvae with rotifers (Planas *et al.*, 2005) enriched with *Phaeobacter* 27-4 reduces the accumulated mortality to the level of the un-challenged control demonstrating an *in vivo* probiotic effect (Planas *et al.*, 2006).

Monitoring of introduced strains in a rearing system is essential to develop protocols and strategies for the industrial application of probiotics. *Phaeobacter* 27-4 has been previously quantified by colony counting based on the (indicative) production of a dark brown pigment (Hjelm *et al.*, 2004a, b). However, as all culture based methods, time is required for colony growth and this impedes the daily monitoring of e.g. the probiont level. Also, to determine the *in vivo* effect of probionts, including sites of action, a continuous monitoring of potential pathogenic bacteria is required. The quantification of pathogenic *Vibrionaceae* by conventional culturing methods is difficult and, for instance, direct counts of *Vibrionaceae* in selective media as TCBS typically underestimate the levels (Mizuki *et al.*, 2006; Munro *et al.*, 1993).

Culture independent methodology could be a suitable alternative to quantify both probiotic and pathogenic bacteria. Real-time PCR can be used as a specific and sensitive method for quantification of bacteria (Klein, 2002) and has been used for detection and quantification of food-borne pathogens (Cai *et al.*, 2006; Davis *et al.*, 2004; Panicker *et al.*, 2004), probiotic bacteria (Bruhn *et al.*, 2006; Kao *et al.*, 2007; Vitali *et al.*, 2003) as well as for marine microbial ecology studies (Skovus *et al.*, 2007). In aquaculture, different real-time PCR protocols have been designed, focusing on detection and quantification of pathogenic vibrios in crustaceans (Goarant and Merien, 2006) or molluscs (Labreuche *et al.*, 2006).

In this chapter, a quantitative real-time PCR protocol was developed, using SYBR Green I as fluorescent dye (Bruhn *et al.*, 2006) to quantify a probiont (*Phaeobacter*) and two pathogens (*L. aguillarum* and *V. splendidus*) in the organisms involved in turbot larval rearing: microalgae (*Isochrysis galbana*), used for the enrichment in fatty acids of the live feed, and in the rotifers (*Brachionus plicatilis*) or *Artemia* used as the feed, as

well as in turbot (*Psetta maxima*) larvae. *L. anguillarum* strain 90-11-287, serotype O1 (Skov *et al.*, 1995) is pathogenic to turbot larvae and, as mentioned, sensitive to the probiont selected. *V. splendidus* DMC-1 was isolated from moribund turbot larvae in the same rearing industry as *Phaeobacter* 27-4 in Galicia (NW Spain) and is also sensitive to *Phaeobacter* 27-4 (Hjelm *et al.*, 2004).

4.2. Materials and methods

4.2.1. Bacterial strains and culture conditions

Phaeobacter (formerly *Roseobacter*, Martens *et al.*, 2006) strain 27-4 was isolated from a Spanish turbot farm (Hjelm *et al.*, 2004a), inhibits *L. anguillarum* and *V. splendidus* (Hjelm *et al.*, 2004a), and is probiotic to turbot larvae (Planas *et al.*, 2006). *L. anguillarum* strain 90-11-287 (serotype O1) and *V. splendidus* strain DMC-1 were isolated from rainbow trout (Skov *et al.* 1995) and from a turbot rearing unit (Hjelm *et al.*, 2004a, Thomson *et al.*, 2005), respectively. Both are pathogenic to turbot larvae (Planas *et al.*, 2005; Thomson *et al.*, 2005).

The strains were cultured in Marine Broth (MB, Difco 2219) at 22 ºC and 110 rpm (*L. anguillarum* and *V. splendidus*) and at 20 ºC under stagnant conditions (*Phaeobacter)*. Pre-cultures were incubated for 72 h and 1 ml inoculated in 100 ml of MB followed by incubation for 24 h (*Vibrionaceae* species) and 48 h (*Phaeobacter*).

4.2.2. Preparation of cultures for quantification

Serial decimal dilutions were prepared from each bacterium in autoclaved seawater (directly taken from *Ría de Vigo* and filtered over 1 μm) and cells harvested at 5 ºC from 1 ml of each dilution at 13,000 × g for 10 min. The cell pellets were washed in 1 ml of autoclaved seawater and harvested (13,000 × g / 5 ºC / 10 min). Bacterial pellets were stored at -20 ºC until use. Bacterial numbers were determined by surface plating on Marine Agar (MA, Difco 2219) and plates were incubated 72 h at 20 ºC.

4.2.3. Microalgae, rotifer, *Artemia* and turbot larvae culturing

The microalgae *Isochrysis galbana* was grown in Conway medium (Walne, 1966) at 20 ºC with continuous light and harvested (5,000 × g / 20 ºC / 10 min) from 200 ml at a concentration of 10^7 cells·ml^{-1}. The pellet was washed in 200 ml of autoclaved seawater, harvested (5,000 × g / 20 ºC / 10 min) and re-suspended in 40 ml of autoclaved seawater. Aliquots of 0.5 ml were dispensed in Eppendorf tubes and harvested (5,000 × g / 20 ºC / 10 min). The pellets, with $2.5 \cdot 10^7$ cells, were stored at -20 ºC until use.

Rotifers (*Brachionus plicatilis*) were grown in autoclaved seawater enriched with *I. galbana* cultured as above. Rotifers concentration was calculated by counting the organisms present in 1 ml of the well-mixed culture, in quadruplicate, on a methacrylate chamber. One million of rotifers was filtered over a nylon mesh with a pore size of 50 μm and re-suspended in 1 l of 1 μm filtered-seawater (10^3 rotifers·ml^{-1}). Fifty millilitres of rotifers solution ($5 \cdot 10^4$ rotifers) were filtered over a nylon mesh of 30 μm of pore size, washed with 0.5 ml of autoclaved seawater and collected in a sterile Eppendorf tube. The tubes were placed into ice for 30 minutes to decant the rotifers to the bottom of the tube and excess of seawater discarded. Samples were stored at -20 ºC until require.

Five-hundred milligrams of *Artemia* cysts (EG, INVE, Belgium) were hatched in 15 l of 1 μm filtered-seawater at 28 ºC, with continuous light and intensive aeration. Hatching was initiated by adding 3 ml of 10 % bleach (Panreac) for 30 min, when the bleach was neutralised by the addition of 3 ml of a thiosulphate (Panreac) solution (125 g·l^{-1}). The nauplii were collected, after 24 h, in a nylon mesh of 100 μm of pore size, washed with tap water for 20 min, re-suspended in 4 l of 1 μm filtered-seawater and counted. The nauplii were collected in a nylon mesh with a pore size of 50 μm, re-suspended in 15 ml of 1 μm filtered-seawater (2500 nauplii·ml^{-1}) and aliquoted in samples of 250 nauplii. All samples were stored at -20 ºC.

Turbot larvae of three days after hatching were supplied by Stolt Sea Farm (Merexo, Spain) and acclimatized and distributed in cylindrical tanks containing 60 litres (35 larvae·l^{-1}) of 1 μm filtered-seawater with *I. galbana* ($1.7 \cdot 10^5$ cells·ml^{-1}). The larvae were fed on the rotifer *B. plicatilis* previously enriched with *I. galbana* for one day. The larvae were collected from the tanks in sterile flasks and anaesthetized with MS222 (Sigma). Subsequently, the larvae were concentrated over a nylon mesh of 250

μm of pore size and washed with sterile seawater. Groups of ten larvae were collected in Eppendorf tubes and re-suspended in 100 μl of autoclaved seawater. The samples were stored at -20 ºC.

4.2.4. Mixing of samples and DNA extraction

Bacterial pellets, corresponding to 10^3 to 10^8 CFU, were mixed with $2.5 \cdot 10^7$ cells of *I. galbana*, 10^4 rotifers, 250 *Artemia* sp nauplii or 10 turbot larvae. Bacteria were added as individual species or as mixtures. All the mixtures were prepared in duplicate with a final volume of 100 μl and the bacterial DNA was extracted (Pintado *et al.*, 2003) adding a cleaning step prior to DNA purification employing the chelating ion resin Chelex 100® (Bio-Rad).

Bacteria were lysed by incubating 1 h at 37 ºC with 200 μl of lysozyme (Sigma, 50 $g \cdot l^{-1}$) and 10 μl of mutanolysine (Sigma, $25 \cdot 10^{-5}$ $U \cdot l^{-1}$) and for 50 min at 50 ºC with 50 μl of N-Lauroylsarcosine (Sigma, 1 %) and 3 μl of proteinase k (Sigma, 10 $g \cdot l^{-1}$). The preparation was cleaned by adding 200 μl of a 36 % Chelex 100® (Bio-Rad) solution and, the samples were boiled for 20 min after incubation (58 ºC, 10 min). The suspension was cooled at room temperature and 100 μl of 9 M ammonium acetate (Panreac) were added, followed by centrifugation (13,000 × g / 10 min / room temperature).The supernatant was transferred to a sterile Eppendorf tube and DNA purified as described before (Pintado *et al.*, 2003), dried in a laminar flow chamber and re-suspended in 50 μl of sterile MilliQ water. All DNAs were frozen (-20 ºC) until analysed.

4.3.5. Design of primers for quantitative real-time PCR

Genes *virA/virB* (Accession number: L08012, Norqvist and Wolf-Watz, 1993) and of haemolysin (Accession number: AM157713, Macpherson, 2004), were selected from *L. anguillarum* and *V. splendidus*, respectively. Ten sets of primers were designed in ten genes described for *Phaeobacter* 27-4 (Geng *et al.*, 2008). All the primers sets were designed using the software Primer 3 Input 4.0 (http://frodo.wi.mit.edu) and synthesized by Sigma-Aldrich (Table 4.1).

Table 4.1. *Phaeobacter* 27-4, *L. anguillarum* 90-11-287 and *V. splendidus* DMC-1 primers tested and used for real-time PCR detection and quantification.

Name	Gene	Primer (R/F)	Start	Tm (ºC)	G+C (%)	Sequence (5'→3')	Accession number	Target
clpR1	*clpX*	R	50	60.00	45	ACAAAATTACCCGCAAGTCG	EF139220	[a] *Phaeobacter* 27-4
clpR2		F	148	60.13	55	CTACGGTGCCTTCCATCAGT		
metR1	*metF*	R	163	59.83	50	GGGTTCGGGTTTGAGTTGTA	EF139218	
metR2		F	258	60.82	55	CTCCACGGTTGAGAAAGACG		
tdaR1	*tdaA*	R	255	60.33	50	GATGATTGGGTCCTTTGCAC	EF139217	
tdaR2		F	335	60.02	50	ATCGCACTGCTTCTTGAGGT		
tdaR3	*tdaB*	R	491	59.98	45	GGGTATCGGATTTCGGATTT	EF139219	
tdaR4		F	591	59.87	50	AGCAGCCATGAATAGCCTGT		
tdaR5	*tdaB*	R	77	59.79	50	AAGCTTTAGCAGCGTTCCAG	EF139212	
tdaR6		F	146	59.67	45	TCGGGCAATATCGAAACTCT		
tdbR1	*tdbA*	R	172	60.27	55	GGGGTCGGGTGGTTAATATC	EF139215	
tdbR2		F	234	59.21	55	AAGCCGACTTGTAGGGTGAC		
tdbR3	*tdbC*	R	861	59.86	55	ACGGTGTCCCTTACCTTCCT	EF139213	
tdbR4		F	931	60.16	55	GCGCTTCTCAAGCACCTAAC		
tdbR5	*tdbD*	R	258	59.55	45	GCACATTGTTGGGAAACTGA	EF139222	
tdbR6		F	333	59.51	45	ATCTTTGGCTCCATCGACAT		
tdbR7	*tdbE*	R	6	59.84	50	AGCACAGGGGTGAGAAAAGA	EF139216	
tdbR8		F	70	59.69	50	AAGGAGGAACGCACTATGGA		
traR1	*traI*	R	212	60.17	40	TCCCCATTTTTCGTGACAAT	EF139221	
traR2		F	281	60.01	50	CAGTGGTGGAAGAACGGTTT		
VA1	*virA/ virB*	R	888	59.28	55	GCACTGTCCGTCATGCTATC	L08012	*L. anguillarum*
VA2		F	986	59.70	40	CATACGCAGCCAAAAATCAA		
VS1	*Splendilysine*	R	465	59.72	55	GTACGTTGGCGAAGACATGA	AM157713	*V. splendidus*
VS2		F	556	59.90	10	CACAGCGATAACCGCTACAA		

[a] *Phaeobacter* 27-4 genes are described in Geng *et al.* (2008)

Specificity of the primer sets was tested using the BLAST search function (http://www.ncbi.nlm.nih.gov). Specificity of the primers was also experimentally checked against the following strains: *Aeromonas hydrophila* (ATCC7966), *Leisingera methylohalidivorans* (DSM 14336), *Marinovum algicola* (DSM 10251), *Phaeobacter gallaeciensis* (DSM 12440), *P. inhibens* T5 and mutant *P. inhibens* T5 (Brinkhoff *et al.*, 2004; Martens *et al.*, 2006), *Roseobacter denitrificans* (DSM 7001), *Sulfitobacter pontiacus* (DSM 10014), *Thalassobius gelatinovorus* (DSM 5887), *V. vulnificus* (NCIMB 2046) and also against *L. anguillarum* 90-11-287 and *V. splendidus* DMC-1. The primer sets designed for both pathogenic *Vibrionaceae* were also checked against *L. anguillarum* (NB10) and *V. splendidus* (NCIMB 1).

In the same way, those primers designed for *Phaeobacter* 27-4 were tested against seven brown-pigmented *Roseobacter* clade strains (five *Phaeobacter* spp [M2-4.4, M3-1.3, M6-4.3, M23-3.1, M30-2.1] and two *Ruegeria* spp [M41-2.2, M43-2.3]) isolated from a Danish turbot farm (Porsby *et al.*, 2008) and fourteen *Roseobacter* clade strains isolated from the same Spanish turbot farm as *Phaeobacter* 27-4 (Hjelm *et al.*, 2004b). Of the fourteen strains, seven were identified by 16S rRNA gene sequence analyses as *Phaeobacter* spp (8-1, 234-9, 234-10, 256-7a, 267-1 and 632-1) or *Ruegeria* spp (270-3) (Porsby *et al.*, 2008).

All bacteria used in specificity test were grown on MA and incubated at 20 ºC for 48 h. DNA was extracted from one colony of each strain and purified by NucleoSpin Tissue kit (Macherey-Nagel) following manufacturer's guidelines.

4.2.6. Quantitative real-time PCR

As the real-time PCR analysis may be affected by PCR inhibitors (Harms *et al.*, 2003), each sample was diluted ten or one-hundred times. Ten microlitres of the diluted target DNA were mixed with the adequate primers (final concentration 4.3 μM) and the *Power* SYBR® Green master mix (Applied Biosystems), containing the AmpliTaq Gold® DNA polymerase, the double strand DNA-binding dye *Power* SYBR® Green and the reference dye ROX®. The quantitative real-time PCR program was performed on a 7500 Fast Real-Time PCR System (Applied Biosystems). PCR amplification was performed as described in Bruhn *et al.* (2006). Dissociation curves were obtained by an additional cycle (15 s at 95 ºC, 1 min at 60 ºC, 15 s at 95 ºC and 15 s at 60 ºC) and used for testing specificity of the PCR products.

4.2.7. Standard curves and quantification of strain mixtures using quantitative real-time PCR

Standard curves relating Ct values to bacterial density ($CFU \cdot ml^{-1}$) were obtained by running DNA extracted from duplicate samples of 10^3 to 10^8 $CFU \cdot ml^{-1}$, alone or in presence of $2.5 \cdot 10^7$ cells of *I. galbana*, 10^4 rotifers, 250 *Artemia* sp nauplii or 10 turbot larvae. The measurements were analysed by using the SDS software, v1.4 from Applied Biosystems.

DNA extracted from duplicate mixtures of known concentrations (10^3 or 10^7 $CFU \cdot ml^{-1}$) of two of the three bacteria, alone or in presence of microalgae, rotifers, *Artemia* or turbot larvae were run in duplicate, with the adequate standard curve and quantified. Turbot rearing organisms without any bacteria added and no template controls (NTC) were also analysed in each real-time PCR.

4.2.8. Quantification by real-time PCR of bacteria experimentally introduced in rotifers and transferred to seawater and turbot larvae

Phaeobacter 27-4, *L. anguillarum* 90-11-287 and *V. splendidus* DMC-1 were bioencapsulated in the rotifer *B. plicatilis* as described in Planas *et al.* (2005, 2006), washed with 1 μm filtered-seawater and transferred to rearing tanks with 60 l of 1 μm filtered-seawater (3 – 5 $rotifers \cdot ml^{-1}$) with *I. galbana* ($1.7 \cdot 10^5$ $cells \cdot ml^{-1}$). Samples of rotifers were taken at the moment of the introduction of rotifers in the rearing tanks (t_0) and at 24 hours (t_{24}). Seawater samples from the rearing tanks were taken before the addition of the rotifers and at 24 hours.

Turbot larvae of three days after hatching were supplied by Stolt Sea Farm (Merexo, Spain), acclimated and fed with rotifers previously loaded with *L. anguillarum* and *Phaeobacter* 27-4 by bioencapsulation as described in Planas *et al.* (2005, 2006). Larvae samples were obtained at the end of a challenge trial (day nine after hatching), after feeding them with rotifers loaded with *Phaeobacter* 27-4 (days three, five and seven post-hatching) and with rotifers loaded with *L. anguillarum* (days four, six and eight post-hatching).

For microbial cultures, rotifers (400 rotifers per sample), larvae (10 larvae per sample) or seawater (10 ml) samples were taken in duplicate and treated as described in

turbot rearing organisms culturing section. Ten-fold dilutions from rotifers or larvae homogenates and of tanks seawater were spread on MA plates and incubated at 20 ºC for three days, using those plates with 30 – 300 colonies for CFU counting. Quantification of *Phaeobacter* 27-4 was done by identification of dark brown pigmented colonies on MA. *Vibrionaceae* were counted by replica-plating of MA plates onto TCBS (Cultimed), as described in Planas *et al.* (2006). The plates were incubated at 20 ºC for 24 h. All culture based analyses were conducted, in duplicate, from two biological replicates.

For quantitative real-time PCR, rotifers (10^4 rotifers per sample) and larvae (10 larvae per sample) samples were treated as described above and the pellets frozen at -20 ºC until DNA extraction. Fifty millilitres of seawater were filtered over a nylon mesh with 30 μm pore size, centrifuged (5,000 × g / 10 min / 20ºC) and washed once with autoclaved seawater. The pellets were frozen at -20 ºC until use. DNA was extracted from duplicate samples of rotifers, larvae or seawater as described in DNA extraction section. Quantification of DNA extracted from duplicate samples was by running a real-time PCR, in duplicate, with the adequate standard curves and set of primers.

4.3. Results

4.3.1. Verification of primers specificity

BLAST testing of the *Vibrionaceae* species primers resulted in unique hits for *L. anguillarum* and *V. splendidus*. In laboratory testing, none of the non-target bacteria were amplified with any of the primers used.

Seven of the ten sets of primers designed for *Phaeobacter* 27-4 were specific for 27-4 when tested with BLAST. However, only primers tdbR3/R4 and traR1/R2 designed in genes encoding for a lytic transglycosylase (*tdbC*) and for a type IV secretion protein (*traI*), respectively, were specific for *Phaeobacter* 27-4 in laboratory testing (Table 4.2). None of the seven strains from the Danish turbot farm gave any amplification whereas five (256-7a, 256-8, 632-1, 658-2, 659-1) of the fourteen strains from the Spanish turbot farm resulted in an amplicon.

The PCR products obtained from the five Spanish strains had the same dissociation curve as *Phaeobacter* 27-4, with melting temperatures of 78 ºC and 75 ºC

for the primers tdbR3/R4 and traR1/R2, respectively. Primers tdbR3/R4 were selected for quantifying *Phaeobacter* 27-4 in turbot rearing samples by real-time PCR, because they gave lower Ct values than traR1/R2 primers (Table 4.2).

Table 4.2. Ct values obtained from specificity test of *Phaeobacter* 27-4 primers against a collection of marine bacteria.

Bacterial strain	Ct value									
	Clp R1/R2	Met R1/R2	Tda R1/R2	Tda R3/R4	Tda R5/R6	Tdb R1/R2	Tdb R3/R4	Tdb R5/R6	Tdb R7/R8	Tra R1/R2
A. hydrophila	[a]-	-	-	-	-	-	-	-	-	-
L.methylhalidovorans	17.82	32.23	-	-	37.69	38.80	-	-	36.59	-
M. algicola	35.09	37.33	36.56	-	-	-	-	-	-	-
Phaeobacter 27-4	12.55	14.69	14.43	14.75	15.57	15.43	14.17	17.73	16.59	15.47
P. gallaeciensis	-	-	-	-	-	-	-	-	38.59	-
P. inhibens T5	30.49	23.51	27.74	24.65	34.83	-	-	28.96	16.93	-
[b]*P. inhibens* T5m	29.15	22.80	27.33	24.04	34.51	-	-	28.68	16.40	-
R. denitrificans	-	-	-	-	-	-	-	-	-	-
S. pontiacus	-	-	-	-	37.64	-	-	-	36.80	-
T. gelationovorus	18.28	-	-	-	-	-	-	-	36.44	-
L. anguillarum	-	-	38.09	-	-	-	-	-	37.28	-
V. splendidus	-	-	-	-	-	-	-	-	-	-
V. vulnificus	-	-	-	-	36.52	-	-	-	-	-

[a] No amplification was observed after 40 cycles; [b] m, mutant strain of *P. inhibens* T5 without pigmentation (Brinkhoff *et al.*, 2004)

4.3.2. Standard curves for quantitative real-time PCR

The DNA extracted from bacterial pellets with 10^3 to 10^8 CFU·ml^{-1} prepared from cultures of *L. anguillarum* (initial level: $5 \cdot 10^9$ CFU·ml^{-1}), *V. splendidus* (10^{10} CFU·ml^{-1}) or *Phaeobacter* 27-4 ($4 \cdot 10^9$ CFU·ml^{-1}) were analysed alone or mixed with $2.5 \cdot 10^7$ cells of *I. galbana*, 10^4 rotifers, 250 *Artemia* sp nauplii or 10 turbot larvae. Samples without any bacteria and a no template control (NTC) were included as negative controls in each run. All standard curves were obtained from the duplicate analysis of DNA extracted from duplicate samples.

The initial amount of DNA in the PCR was quantified by using Quant-It™ Picogreen® dsDNA Reagent and Kits (Invitrogen) and gave a medium value of 78 ±

4.18 ng. Real-time PCR was initially performed on un-diluted samples, but this caused inhibition of the PCR reaction. Subsequently, all samples were 10-fold diluted, except those samples containing microalgae, which were diluted 100-fold.

No amplification was detected in any of the negative controls or NTC. Linear standard curves were obtained comparing Ct values from real-time PCR with CFU·ml^{-1} for all mixtures containing *Phaeobacter* 27-4 (Figure 4.1) with correlation coefficients (R^2) between 0.96 – 0.98. However, the y-intercept of the standard curves differed depending on the background (microalgae, larvae etc.) into which *Phaeobacter* cells were mixed (Figure 4.1). Also, standard curves obtained for *L. anguillarum* and *V. splendidus* were linear with R^2 = 0.93 – 0.94 and R^2 = 0.97 – 0.99, respectively. As for *Phaeobacter*, the standard curves varied slightly depending on the background mixture. The parallel shift (order) of the standard curves, from the highest to the lowest Ct value and independently of the bacterial strain added, was: microalgae, rotifers, larvae and *Artemia* nauplii (Figure 4.1).

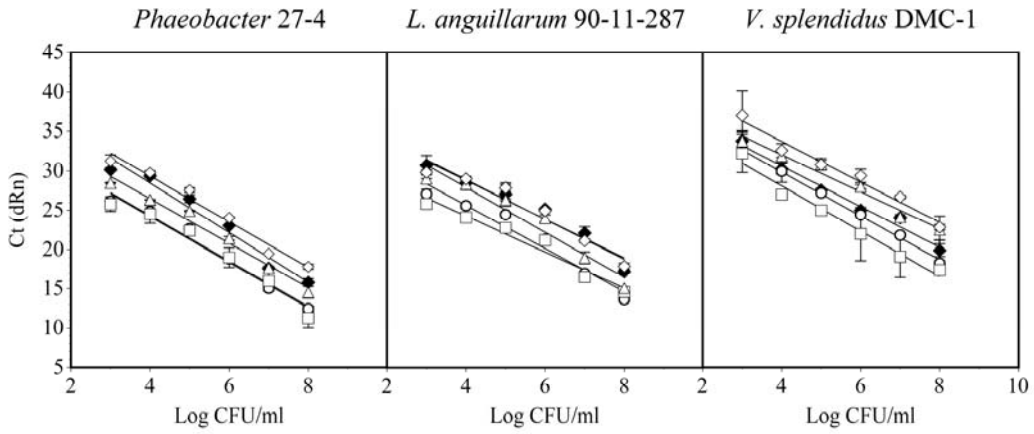

Figure 4.1. Standard curves of *Phaeobacter* 27-4, *Listonella anguillarum* 90-11-287 and *Vibrio splendidus* DMC-1 alone (♦) or in presence of microalgae (◊), rotifers (Δ), *Artemia* (□) or turbot larvae (○). Each standard curve was obtained by running, in duplicate, DNA extracted from two samples (n=4) and standard deviations are also shown.

It was determined whether the variability on the standard curves dependent on background material was a random variable or varied systematically. Three cultures of each bacterium were prepared as described in Materials and Methods section. Standard curves relating Ct values with CFU·ml^{-1} of each culture were obtained by running three independent assays of the DNA extracted from duplicate samples. Standard deviations obtained from y-intercept were around one cycle for the three cultures analysed of each

bacterium, except for two cultures of *L. anguillarum* that showed standard deviations of three cycles (Table 4.3). The effect of background material on the parallel shift of the standard curve was reproducible and consistent with respect to the type of background material.

Table 4.3. Standard curves obtained from DNA extracted from duplicate samples of *Phaeobacter* 27-4, *Listonella anguillarum* 90-11-287 and *Vibrio splendidus* DMC-1 pure cultures running in triplicate. Values are mean and standard deviation of three independent assays. Each assay value is the average of a technical replicate.

Strain	Trial no	Slope (Mean ± StDev)	Y-intercept (Mean ± StDev)	R^2 (Mean ± StDev)
Phaeobacter 27-4	1	-3.06 ± 0.02	39.06 ± 0.94	0.94 ± 0.01
	2	-3.06 ± 0.05	39.16 ± 0.89	0.94 ± 0.00
	3	-2.88 ± 0.02	38.11 ± 0.76	0.95 ± 0.00
L. anguillarum 90-11-287	1	-2.21 ± 0.65	34.69 ± 3.14	0.94 ± 0.04
	2	-2.78 ± 0.03	38.48 ± 0.88	0.98 ± 0.00
	3	-3.13 ± 0.26	40.11 ± 2.61	0.97 ± 0.01
V. splendidus DMC-1	1	-2.76 ± 0.20	41.98 ± 0.57	0.97 ± 0.02
	2	-1.94 ± 0.10	35.66 ± 0.79	0.95 ± 0.02
	3	-2.55 ± 0.13	39.42 ± 1.12	0.96 0.02

4.3.3. Real-time PCR quantification with known concentrations of the target bacteria

DNA extracted from duplicate mixtures of *Phaeobacter* 27-4, *L. anguillarum* 90-11-287 and *V. splendidus* DMC-1 as pure cultures or in a background mixture of different turbot rearing organisms was quantified by real-time PCR, in duplicate, at the same time as the corresponding standard curve and using the adequate set of primers (Table 4.4). No amplification was observed in samples of microalgae, *Artemia*, rotifers and turbot larvae without any bacteria added.

Real-time PCR analysis of mixtures with 10^7 CFU·ml^{-1} of *Phaeobacter* 27-4 accurately estimated the number using the standard curves of the appropriate mixture described above.

Table 4.4. Quantification of *Phaeobacter* 27-4, *L. anguillarum* 90-11-287 or *V. splendidus* DMC-1 in mixtures with known concentrations of target bacterium and different turbot rearing organisms. Values are mean and standard deviation of two independent assays. Each assay value is the average of a technical replicate.

Bacteria added (Log CFU·ml^{-1})	[a] Turbot organisms	Estimated Log CFU·ml^{-1}		
		L. anguillarum	*V. splendidus*	*Phaeobacter*
L. anguillarum (7) *V. splendidus* (3)	None	7.35 ± 0.04	3.03 ± 0.20	[b] -
	Artemia	5.37 ± 0.00	2.16 ± 0.36	-
	Microalgae	4.84 ± 0.66	2.85 ± 0.65	-
	Turbot larvae	6.67 ± 0.35	2.54 ± 0.27	-
	Rotifers	6.03 ± 0.02	3.06 ± 0.47	-
L. anguillarum (7) *Phaeobacter* (3)	None	7.03 ± 0.83	-	3.63 ± 0.15
	Artemia	5.48 ± 0.06	-	2.42 ± 0.13
	Microalgae	5.70 ± 0.11	-	3.31 ± 0.64
	Turbot larvae	6.58 ± 0.41	-	3.86 ± 0.72
	Rotifers	6.09 ± 0.11	-	3.31 ± 0.64
L. anguillarum (3) *V. splendidus* (7)	None	2.84 ± 0.69	7.35 ± 0.32	-
	Artemia	2.82 ± 0.64	5.96 ± 0.33	-
	Microalgae	1.30 ± 0.28	8.62 ± 0.07	-
	Turbot larvae	1.09 ± 0.51	6.96 ± 0.22	-
	Rotifers	2.59 ± 0.07	7.20 ± 1.36	-
L. anguillarum (3) *Phaeobacter* (7)	None	2.18 ± 0.08	-	7.54 ± 0.09
	Artemia	2.04 ± 0.29	-	6.90 ± 0.13
	Microalgae	1.82 ± 0.09	-	7.77 ± 0.05
	Turbot larvae	3.37 ± 0.01	-	7.34 ± 0.08
	Rotifers	2.01 ± 0.00	-	7.52 ± 0.04
V. splendidus (7) *Phaeobacter* (3)	None	-	6.40 ± 0.28	3.94 ± 0.02
	Artemia	-	4.91 ± 0.14	3.00 ± 0.39
	Microalgae	-	8.36 ± 0.08	3.24 ± 0.17
	Turbot larvae	-	6.59 ± 0.58	2.95 ± 0.31
	Rotifers	-	7.59 ± 0.74	2.69 ± 0.26
V. splendidus (3) *Phaeobacter* (7)	None	-	2.00 ± 0.81	7.55 ± 0.08
	Artemia	-	2.00 ± 0.36	6.85 ± 0.13
	Microalgae	-	3.07 ± 0.71	7.57 ± 0.04
	Turbot larvae	-	2.97 ± 0.62	7.20 ± 0.22
	Rotifers	-	3.22 ± 0.46	7.77 ± 0.03

[a] Quantities of turbot rearing organisms in each mixture were: 250 *Artemia* nauplii, $2.5 \cdot 10^7$ cells of microalgae, 10 turbot larvae and 10^4 rotifers; [b] Not analysed.

Except for one sample, levels of 10^3 CFU·ml^{-1} were also accurately estimated. Similarly, real-time PCR correctly estimated the number of *L. anguillarum* in the mixtures with 10^7 CFU·ml^{-1} except for mixtures containing *Artemia* nauplii, microalgae or rotifers in which the level was under-estimated (10^5-10^6 CFU·ml^{-1}).

For the mixtures with 10^3 CFU·ml^{-1} of *L. anguillarum*, the extrapolated quantity was 10^3 CFU·ml^{-1} when mixed with *V. splendidus* alone, in a background matrix of *Artemia* or rotifers, as in presence of turbot larvae plus *Phaeobacter* 27-4; 10^2 CFU·ml^{-1} in mixtures with *Phaeobacter* 27-4 pure cultures as in presence of *Artemia*, rotifers or microalgae and 10 CFU·ml^{-1} in presence of microalgae or turbot larvae plus *V. splendidus*.

Real-time PCR estimation of mixtures containing 10^7 CFU·ml^{-1} of *V. splendidus* in presence of *Artemia* was variable resulting in estimates between 10^5 and 10^6 CFU·ml^{-1} and those in presence of microalgae gave extrapolated quantities of 10^8 CFU·ml^{-1}. The mixtures containing 10^3 CFU·ml^{-1} of *V. splendidus* were estimated to contain 10^3 CFU·ml^{-1} by real-time PCR for all the samples except when mixed with 10^7 CFU·ml^{-1} of *Phaeobacter* 27-4 and in presence of *Artemia* nauplii, where 10^2 CFU·ml^{-1} was the estimated value.

4.3.4. Real-time PCR quantification of target bacteria experimentally introduced in rotifers (bioencapsulation) and transferred to seawater and turbot larvae in a challenge trial

The real-time PCR method was used to study the bioencapsulation and maintenance of *Phaeobacter* 27-4, *L. anguillarum* 90-11-287 and *V. splendidus* DMC-1 in rotifers. Samples from rotifers were taken after the period of bioencapsulation (t_0) and 24 hours after being transferred to a rearing tank and kept in turbot larval rearing conditions (Table 4.5). Bacteria transferred from the rotifers to the seawater of rearing tanks were also analysed (Table 4.5). Results of real-time PCR and culture methods were compared.

Phaeobacter 27-4 bioencapsulated in rotifers constituted 12 % of the cultivable bacteria. Once rotifers were transferred to tanks, the proportion of the probiotic strain represented 2.3 % of total bacteria in rotifers and 0.7 % of total bacteria in seawater. The bioencapsulated *L. anguillarum* accounted for 12 % of the total bacterial load.

When rotifers loaded with this bacterium were transferred to rearing tanks and maintained for 24 h, the proportion of *L. anguillarum* in the rotifer decreased to 3 % and was only 0.3 % of the bacteria present in the surrounding seawater. *V. splendidus* accounted for 1.9 % of the bacteria in bioencapsulated rotifers and 24 h after the transfer of rotifers to rearing tanks it was not detected, by plating, in rotifers or seawater.

Table 4.5. Quantification of *Phaeobacter* 27-4, *L. anguillarum* 90-11-287 and *V. splendidus* DMC-1 bioencapsulated in rotifers and present in seawater. Values are mean and standard deviation of two independent assays. Each assay value is the average of a technical replicate.

Strains	Time (h)	Log CFU·rotifer^{-1}		Log CFU·ml^{-1} seawater	
		[a] Plate Count	QRT-PCR	[a] Plate Count	QRT-PCR
Phaeobacter	[b] 0	1.99 ± 0.00	2.43 ± 0.04	ND	ND
	24	< 1 CFU	< 1 CFU	4.15 ± 0.21	4.50 ± 0.11
L. anguillarum	[b] 0	2.38 ± 0.30	4.62 ± 0.31	ND	ND
	24	2.49 ± 0.55	2.13 ± 0.58	4.00 ± 0.99	3.94 ± 0.29
V. splendidus	[b] 0	1.69 ± 0.02	1.49 ± 0.72	ND	ND
	24	ND	< 1 CFU	ND	5.63 ± 0.12

[a] *Phaeobacter* 27-4 was quantified on MA and *Vibrionaceae* by replica plating of MA plates on TCBS; [b] Bacterial concentration after bacterial bioencapsulation; ND: Not detected.

In a subsequent challenge trial, turbot larvae were fed alternatively with rotifer bioencapsulated with *Phaeobacter* 27-4 or *L. anguillarum* 90-11-287. Both, the probiotic and the pathogen were detected by real-time PCR at the end of the experiment (day nine after hatching), showing concentrations of 47 ± 2.40 CFU·larva^{-1} and (1.3 ± 1.12)·10^{4} CFU·larva^{-1}, respectively. However, *Phaeobacter* 27-4 was not detected by plate count and *L. anguillarum* concentration (95 ± 1.02 CFU·larva^{-1}) was two logarithmic units below the one estimated by real-time-PCR.

4.4. Discussion

Tracking the presence and quantity of probiotic bacteria introduced into a system (a host) is important when evaluating its effectiveness and site of action. This can be difficult by growth based culture analyses due to the associate microbiota and the complex matrix of the samples.

In the present study it has been demonstrated that a specific and sensitive method for quantifying both a probiont and the target pathogenic agents can be developed based on real-time PCR. This real-time PCR can be used to quantify the organisms in challenge trials containing all the components of a turbot larval rearing system. The monitoring and quantification of both probiotic and pathogenic bacteria in the *in vivo* trials will help in the development of application strategies and protocols, as e.g. bioencapsulation in live prey. It was also demonstrated in this study that a single universal standard curve cannot be used, being essential to prepare standard curves (relating CFU to Ct) in mixtures representative of the environment in which the real-time PCR method is to be used.

The 16S rRNA gene has been used to design specific primers for real-time PCR (Kao *et al.*, 2007; Vitali *et al.*, 2003) but primers previously designed within the *Phaeobacter* 27-4 16S rRNA gene were not specific (Bruhn *et al.*, 2006). Genes involved in the synthesis of tropodithietic acid (TDA) by *Phaeobacter* 27-4 (Bruhn *et al.*, 2005) have been identified (Geng *et al.*, 2008), and these genes were used for primer design. These primers will not be strain specific as other TDA-producing bacteria, as *P. inhibens* T5, were also amplified as were one third of similar strains from the Spanish hatchery (Hjelm *et al.*, 2004b). Although not tested during *in vivo* challenge trials, it would be anticipated that these strains, due to TDA production, would also have a probiotic potential.

Primers designed in the haemolysin region have been used to detect *Vibrionaceae* species (Davis *et al.*, 2004; Panicker *et al.*, 2004). In the present work, for *V. splendidus* DMC-1 specific primers were successfully designed within a gene related to haemolytic activity (Macpherson, 2004). In *L. anguillarum* several genes are responsible of haemolytic activity (Rock and Nelson 2006), and we therefore tried to design primers in other genes to improve primers specificity. Specific primers for *L. anguillarum* 90-11-287 were successfully designed within the gene *virA/virB*, which encodes for a major surface antigen that is essential for the virulence of serotype O1 (Norqvist and Wolf-Watz, 1993).

Classic phenol-chloroform protocol preceded by a cleaning step using the chelating ion resin Chelex® 100 eliminates proteins, low-molecular-weight ionic moieties (Ochert *et al.*, 1994) and polysaccharides (Poli *et al.*, 1993) giving high quality DNA extracted from complex matrices, as those from the organisms involved in turbot rearing. Inhibitory agents of polymerase are present in seawater (Kirshtein *et al.*, 1993)

and, as all samples were prepared and washed with autoclaved seawater, the AmpliTaq® Gold polymerase could be inhibited (Al-Soud and Rådström, 1998), however, out dilution of samples have likely eliminated this inhibition. Microalgae, as *I. galbana*, are rich in polysaccharides (Brown, 1991) which can inhibit the PCR (Ochert *et al.*, 1994; Poli *et al.*, 1993), explaining that samples containing microalgae had to be diluted ten times more than other samples. Similarly, Park *et al.* (2007) found that to eliminate inhibitors of the PCR present in DNA extracted by a commercial kit from microalgae, samples had to be diluted one-hundred-fold, even when samples were chemically treated.

The detection limit of the real-time PCR was similar to the results previously obtained for *Phaeobacter* 27-4 (Bruhn *et al.*, 2006) and *Vibrionaceae* (Cai *et al.*, 2006; Goarant and Merien, 2006). The slopes of the different standard curves were very similar, but the y-intercept varied depending on the background matrix. Hierro *et al.* (2006) also observed y-intercept variations of the standard curves obtained from yeast strains when grown on different wines. Variations on y-intercept of pure cultures standard curves in three independent assays were around one cycle (Table 4.3), being similar as variations observed between the standard curves from the same bacteria in presence of different background matrix (Figure 4.1). This would suggest that variability could be assumed as inherent to the method and that a pure culture standard curve would be suitable to quantify the samples in the different matrix. However, the effect of a particular background organism (microalgae, rotifers, etc) was the same in three independent assays. Hence, to verify the possible interactions of the matrix in the method and to establish the accuracy of the quantification, standard curves from the target bacteria with the correspondent organism in which it will be analysed must be prepared.

Phaeobacter 27-4 was accurately quantified in all artificial mixtures by real-time PCR but *L. anguillarum* and *V. splendidus* concentration in some of the samples was lower than plate counts. Panicker *et al.* (2004) have explained similar results by the presence of PCR inhibitors in shellfish homogenates but our results can not be explained by the presence of PCR inhibitors as *Phaeobacter* 27-4 was always correctly amplified.

The bioencapsulation levels and the resident time in rotifers for *Phaeobacter* 27-4 and *L. anguillarum* 90-11-287 were similar to those previously obtained (Planas *et al.*, 2005, 2006).

Naturally occurring *Vibrionaceae* can be difficult to culture (Huq and Colwell, 1995). Similarly to previous studies (Cai *et al.*, 2006; Zhou *et al.*, 2007), a higher concentration of target *Vibrionaceae* was estimated by real-time PCR than by culturing. This could be due to the presence of viable but not cultivable (VBNC) (Cai *et al.*, 2006) or dead bacteria (Cai *et al.*, 2006; Zhou *et al.*, 2007). The different detection limit and the low proportion of the target bacteria can also explain the differences between the culture and PCR based methods. Quantification by culture dependent techniques may be difficult in a system, e.g. fish larvae rearing units, where antagonistic and pathogenic strains are coexisting in low levels as compare to total bacteria. Real-time PCR can avoid those problems.

In conclusion, the developed quantitative real-time PCR protocol can be used to detect and quantify the probiotic bacteria *Phaeobacter* 27-4 and the pathogens *L. anguillarum* 90-11-287 and *V. splendidus* DMC-1 in trials with rotifers or in turbot larval challenges. As the background sample material may influence the standard curve relating Ct to $CFU \cdot ml^{-1}$, it is important that separate standard curves be prepared for each scenario.

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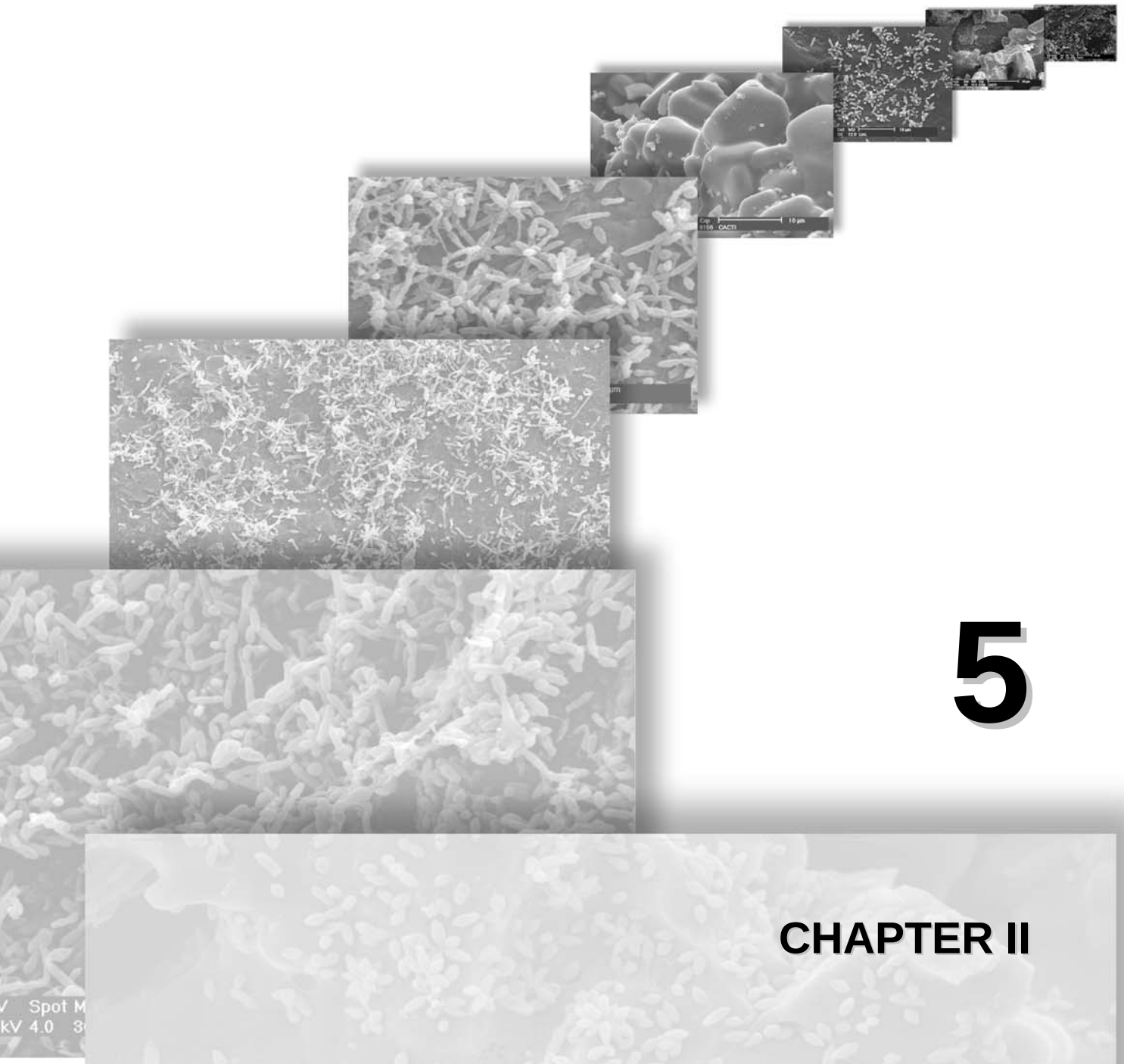

5

CHAPTER II

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5. CHAPTER II

Different colonization and residence time of *Listonella anguillarum* and *Vibrio splendidus* in the rotifer *Brachionus plicatilis* determined by real-time PCR and DGGE

5.1. Introduction

Rotifers (*Brachionus plicatilis*) and *Artemia* are essential live prey in larval rearing of marine fish species. Rotifers, commonly used as the first prey in larvae artificial food chain, are major carriers of bacteria (Munro *et al.*, 1993). Most of bacteria in rotifers are not pathogenic but detrimental effects on fish larvae can be caused by the accumulation of bacteria in prey (Dhert, 1996). Bacteria associated with rotifer cultures have been related to unexpected mortalities or to suppressed growth in rotifers (Yu *et al.*, 1990), as well as to low survival and growth in fish larvae (Gatesoupe, 1989; Nicolas *et al.*, 1989; Pérez-Benavente and Gatesoupe, 1988).

The genus *Vibrio* has been found to be dominant in rotifers (Verdonck *et al.*, 1997) and several *Vibrio* species has been reported to cause high mortality episodes in the culture of some fish species (Thomson *et al.*, 2005; Toranzo *et al.*, 1995). Fish pathogens *Listonella anguillarum* and *Vibrio splendidus* have been found associated with live prey (Thomson *et al.*, 2005; Verdonck *et al.*, 1997), causing mortality to fish larvae (Reid *et al.*, 2009; Sandlund and Bergh, 2008; Thomson *et al.*, 2005). The strains *L. anguillarum* 90-11-287 and *V. splendidus* DMC-1 are pathogenic to turbot larvae but the infection patterns for both species seem to be different. *V. splendidus* was detected and isolated from the gut of diseased larvae (Reid *et al.*, 2009; Thomson *et al.*, 2005) whereas *L. anguillarum* was found in the epidermis of experimentally infected turbot larvae (Planas *et al.*, 2005).

Different infection patterns of pathogenic *Vibrionaceae* bacteria could be dependent on the chemotaxis and adhesion capacity to mucosal surfaces (skin, gill, intestine) (Balebona *et al.*, 1995, Bordas *et al.*, 1998) but also on the entry route (prey or seawater) into fish (Grisez *et al.*, 1996; Muroga *et al.*, 1990; Planas *et al*, 2005). These

factors, among others, would determine which organs would be infected (Grisez *et al.*, 1996; Olsson *et al.*, 1996; Villamil *et al.*, 2003).

Transference of pathogens from live prey to target organisms can be performed directly, by ingestion of the prey, and/or indirectly, by release of pathogens from prey to seawater. Therefore, colonization of intestine and adhesion to external epithelium (gill, skin) could be determined by the way pathogens and target organisms come in contact.

Several models for experimental infection of fish larvae have been developed based on challenge trials with *Vibrionaceae* bacteria, by bioencapsulation of bacteria in rotifers or *Artemia* and administration of the infected live prey to turbot larvae (Grisez *et al.*, 1996; Planas *et al.*, 2005). However, colonization and maintenance of pathogenic strains in live prey or displacement of microbiota associated with prey have not been previously studied. This information would be valuable on the development of experimental infection models or protocols but also on the identification of potential ways of entry (prey, seawater or both) of pathogens into target organisms. All those factors are also determinants to design strategies for infection control in fish larviculture.

Monitoring of introduced pathogens requires the use of specific strain detection techniques. Traditionally, quantification of pathogenic *Vibrionaceae* has been conducted by culture based methods. These methods may underestimate the concentration of *Vibrionaceae* bacteria (Mizuki *et al.*, 2006; Munro *et al.*, 1993) which can be also biased or hindered by the presence of non introduced *Vibrionaceae* naturally present in rotifers (Thomson *et al.*, 2005; Verdonck *et al.*, 1997). Furthermore, specificity of TCBS is questionable as it has been demonstrated that other bacteria different to *Vibrionaceae* can grow in this medium (López-Torres and Lizárraga-Partida, 2001). The use of real-time PCR methods, as the recently developed for specific quantification of *L. anguillarum* 90-11-287 and *V. splendidus* DMC-1 (Prol *et al.*, 2009), avoids these constraints.

Another important aspect not considered before is the study of displacement or modification of autochthonous bacterial populations due to the introduction of pathogens. Culture dependent techniques are time consuming and underestimate microbial diversity as less than 1 % of total bacteria in natural seawater systems are cultivable (Hansen and Olafsen, 1999). The study of autochthonous microbiota modifications in live prey due to introduction of pathogens is more accurate with

application of culture independent genetic fingerprinting techniques, such as Denaturing Gradient Gel Electrophoresis (DGGE). DGGE is a reliable and rapid method to study the variation of dominant bacteria and to characterize complex microbial populations (Muyzer *et al.*, 1993). Bacterial community fingerprints have been previously obtained from rotifers (Rombaut *et al.*, 2001; Qi *et al.*, 2009) and several aquaculture systems by using DGGE (Griffiths *et al.*, 2001; McIntosh *et al.*, 2008; Sandaa *et al.*, 2003) being most of studies focussed on seasonal variations. Recently, DGGE has been used to evaluate changes caused by different probiotic strains in rotifers microbial community composition (Qi *et al.*, 2009).

This chapter is focussed on two pathogenic strains, *Listonella anguillarum* 90-11-287 and *Vibrio splendidus* DMC-1, differing in their infection characteristics. Colonization and residence capabilities of both pathogens in rotifers (*Brachionus plicatilis*) were analysed by specific quantitative real-time PCR. The effect of infection on bacterial community associated with rotifer cultures was analysed by PCR-DGGE.

5.2. Materials and methods

5.2.1. Bacterial strains and culture conditions

The selected pathogens *Listonella anguillarum* strain 90-11-287 (serotype O1) (Skov *et al.*, 1995) and *Vibrio splendidus* strain DMC-1 (Hjelm *et al.*, 2004a; Thomson *et al.*, 2005) demonstrated to be pathogenic for turbot larvae (Planas *et al.*, 2005; Thomson *et al.*, 2005) and a model for experimental infection has been successfully developed for *L. anguillarum* 90-11-287 (Planas *et al.*, 2005).

The strains were routinely cultured in Marine Broth (MB, Difco 2219) at 22 ºC and 110 rpm. Pre-cultures were incubated for 72 h and 1 ml inoculated in 100 ml of MB, followed by incubation for 24 h and sub-cultured once under the same conditions.

5.2.2. Infection of rotifers with the pathogens

The rotifer *Brachionus plicatilis* was routinely fed on the yeast *Saccharomyces cerevisiae* and enriched with the microalgae *Isochrysis galbana* for 24 h. *I. galbana* was grown in Conway medium (Walne, 1966) at 20 ºC and under continuous light.

Colonization of rotifers with *L. anguillarum* 90-11-287 or *V. splendidus* DMC-1 was performed according to the bioencapsulation protocol described by Planas *et al.* (2005). One million of enriched rotifers were filtered over a nylon mesh of 30 μm pore size and re-suspended in 1 l of 1-μm-filtered seawater. Two-hundred millilitres of a 24 h culture of *L. anguillarum* (10^9 CFU·ml^{-1}) or *V. splendidus* ($2{\cdot}10^{10}$ CFU·ml^{-1}) were added to rotifer cultures (200 rotifers·ml^{-1}) in a volume adjusted to 5 l of 1-μm-filtered seawater. After 3 h, rotifers from each fish pathogen suspension were filtered over a nylon mesh of 50 μm pore size, washed and re-suspended in 1 l of 1-μm-filtered seawater (10^3 rotifers·ml^{-1}).

Maintenance of pathogens in rotifers was assayed under conditions similar to those used in turbot larval rearing and as described by Planas *et al.* (2006). Rotifers freshly colonized by *L. anguillarum* or *V. splendidus* were transferred (c2.5 rotifers·ml^{-1}) and maintained for 96 h in 60 l larvae rearing tanks filled with 1-μm-filtered seawater containing a suspension of *I. galbana* ($2{\cdot}10^5$ cells·ml^{-1}). Tanks will be referred as Treatment L and Treatment V depending on whether rotifers were infected with *L. anguillarum* or *V. splendidus*, respectively. Tanks containing rotifers enriched with *I. galbana* and with no added pathogens were used as controls (Treatment Control). All situations were conducted in duplicate and partial renovation (30 – 40 %) of tanks seawater was applied in alternate days, including the proportional addition of microalgae. Rotifer counts were done daily in each tank.

5.2.3. Preparation of samples for microbiological analysis

After infection of rotifers and transfer to rearing tanks, samples from both rotifers and seawater were taken at 0, 3, 6, 24, 48, 72 and 96 h. All analyses were conducted in duplicate.

Four-hundred (for plate-counting) or one million (for DNA extraction) rotifers were filtered over a nylon mesh of 30 μm pore size, washed with 0.5 ml of autoclaved seawater, collected in an Eppendorf tube and placed in ice for 30 min to facilitate rotifers decantation. Excess seawater was discarded and the final volume adjusted to 0.1 ml. Rotifers were then homogenized using an Eppendorf micropestle and the final volume adjusted to 0.5 ml with autoclaved seawater.

Five millilitres of seawater from each tank were filtered over a nylon mesh of 30 μm pore size, and the filtrate used for plate counting. For DNA extraction, 50 ml samples of 30-μm-filtered seawater were centrifuged (5,000 × g / 10 min / 20 ºC), the resultant pellet re-suspended in 1 ml of autoclaved seawater and centrifuged again (5,000 × g / 10 min / 20 ºC). The final pellet was stored at -20 ºC until DNA extraction.

Serial ten-fold dilutions from rotifer and seawater samples were prepared in autoclaved seawater and then plated on Marine Agar (MA, Difco 2219). Incubation was conducted in the dark at 20°C for 5 days. Plates containing 30 – 300 colonies were selected for CFU counts and further isolation of morphologically different colonies with exception of introduced strains. Plates were then replicated on Thiosulfate Citrate Bile Sucrose (TCBS, Cultimed) agar plates (Planas *et al.*, 2005) and incubated for 24 – 48 h at 20 ºC.

5.2.4. DNA extraction

DNA was extracted from one million rotifers, fifty millilitres of seawater or one colony of the different bacterial morphotypes with a Phenol: Chloroform: Isoamyl Alcohol method (Pintado *et al.*, 2003). Bacteria were lysed by incubating 1 h at 37 ºC with 200 μl of lysozyme (Sigma, 50 $g \cdot l^{-1}$) and 10 μl of mutanolysine (Sigma, $25 \cdot 10^{-5}$ $U \cdot l^{-1}$) and for 50 min at 50 ºC with 50 μl of N-Lauroylsarcosine (Sigma, 1 %) and 3 μl of proteinase k (Sigma, 10 $g \cdot l^{-1}$). The preparation was cleaned by adding 200 μl of a 36 % Chelex 100® (Bio-Rad) solution and, the samples were boiled for 20 min after incubation (58 ºC, 10 min). The suspension was cooled at room temperature and 100 μl of 9 M ammonium acetate (Panreac) were added, followed by centrifugation (13,000 × g / 10 min / room temperature). The supernatant was transferred to a sterile Eppendorf tube and DNA purified as described before (Pintado *et al.*, 2003), dried in a laminar flow chamber and re-suspended in 50 μl of sterile MilliQ water. All DNAs were frozen (-20 ºC) until analysed.

5.2.5. Quantitative real-time PCR

The primers VA and VS were used for real-time PCR (Table 5.1). These primers were designed within genes encoding for virulence, being specific for *L. anguillarum* 90-11-287 (VA) and *V. splendidus* DMC-1 (VS) (Prol *et al.*, 2009).

Standard curves were prepared in presence of the appropriate background organism (rotifers or microalgae) and established a correlation between Ct (Cycle threshold) and CFUs in samples obtained from 48 h bacterial cultures. In those conditions, it was assumed that CFUs corresponded to the number of target bacteria. Those standard curves were used to calculate the number of target bacteria in the samples.

One-hundred nanograms of ten-fold (rotifers) or one-hundred-fold (seawater) diluted DNA were mixed with the adequate primers (final concentration 4.3 μM) and the *Power* SYBR® Green master mix (Applied Biosystems), containing the AmpliTaq Gold® DNA polymerase, the double strand DNA-binding dye *Power* SYBR® Green and the reference dye ROX®. The real-time PCR program was performed on a 7500 Fast Real-Time PCR System (Applied Biosystems). PCR amplification was performed as described in Prol *et al.* (2009). Dissociation curves were used to test the specificity of obtained PCR products. No template DNA controls (NTC) and samples of the appropriate background matrix without any bacteria added were used as negative controls in each run.

5.2.6. PCR-DGGE

Purified DNA was amplified with primers gc338f and 518r (Table 5.1) spanning the V3 region of the 16S rDNA (Muyzer *et al.*, 1993). Amplification was performed in a GeneAmp 2700 PCR System (Applied Biosystems) thermal cycler. One-hundred nanograms of DNA were mixed with each primer (0.25 μM), the deoxynucleotides triphosphate mix (dNTPs) (0.2 mM), $MgCl_2$ (1.5 mM), PCR buffer for *Taq* polymerase (1X), *Taq* polymerase (0.05 $U \cdot \mu l^{-1}$) and BSA (0.4 mM) in a final volume of 50 μl. PCR consisted in a total of thirty-one cycles. Activation of *Taq* polymerase was by one cycle at 94°C for five min followed by twenty cycles with three steps (94 ºC for 1 min, 55 ºC for 1 min, 72 ºC for 3 min). A "touchdown" PCR was performed to increase amplification specificity and to reduce the formation of spurious by-products (Muyzer *et al.*, 1993). The annealing temperature was set 10 °C above the expected annealing temperature (55°C) and decreased by 0.5 °C every second step of the twenty cycles, followed by ten additional cycles (94 °C for 1 min, 55 °C for 1 min and 72 for °C 3 min). A final extension was performed at 72 °C for 10 min. The amplification products were analysed first by electrophoresis in 2 % agarose gels and quantified by using the Hyperladder IV (Bio-Rad) ladder and Quantity One software (Bio-Rad).

Table 5.1. Primers and their respective sequences used for PCR-DGGE, real-time PCR and sequencing.

Application	Primer	Sequence (5'- 3')	Reference
Real-time PCR	VAf	CATACGCAGCCAAAAATCAA	Prol *et al.* (2009)
	VAr	GCACTGTCCGTCATGCTATC	
	VSf	CACAGCGATAACCGCTACAA	
	VSr	GTACGTTGGCGAAGACATGA	
PCR-DGGE	gc338f*	ACTCCTACGGGAGGCAGCAG	Muyzer *et al.* (1993)
	518r	ATTACCGCGGCTGCTGG	
Sequencing	27f	AGAGTTYGATCMTGGCTCAG	Lane (1991)
	907Mr	CCGTCAATTCMTTTRAGTTT	
	338f	ACTCCTACGGGAGGCAGCAG	
	518r	ATTACCGCGGCTGCTGG	Muyzer *et al.* (1993)

*A GC clamp was attached to the 5' end of primer 338f to obtain gc338f (GC clamp, 5'CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG)

Five hundred nanograms of the PCR products were analysed by DGGE in a Bio-Rad DCode following the procedure described by Muyzer *et al.* (1993). Samples were loaded on 8 % (w/v) polyacrylamide gels in 1X TAE. All parallel electrophoreses were performed at 60 °C using gels containing a 30 to 60 % gradient UF (100 % corresponded to 7 M urea and 40 % [v/v] formamide) increasing in the direction of electrophoresis. Gels were run at 20 V for 10 min, followed by 3 h at 200 V and stained by bathing for 30 min in a 0.5 % (v/v) ethidium bromide solution and rinsed for 30 min in distilled water.

5.2.7. Analysis of DGGE profiles

The obtained DGGE profiles were subsequently processed using Quantity One v4.4.1 software package (Bio-Rad) and three parameters were calculated to analyse the influence of *L. anguillarum* 90-11-287 and *V. splendidus* DMC-1 in rotifer bacterial community: range-weighted richness (*Rr*), dynamics (*Dy*) and functional organization (*Fo*) (Marzorati *et al.*, 2008).

Range-weighted richness (*Rr*) relates the number of bands present in the DGGE profile to the percentage of denaturing gradient needed to describe the total diversity of

the sample analysed. This index was calculated at all times analysed (0-96 h) to determine the influence of introduced strains in the carrying capacity of the system.

Dynamics of the microbial community (*Dy*) can be interpreted as the number of species that on average come to significant dominance (above the detection limit of the technique) at a given habitat during a defined time interval. Dynamics can be calculated using the comparison tool moving-window analysis. A matrix of similarities for the densiometric curves of the bands profiles was calculated based on the Pearson product-moment correlation coefficients employing STATISTICA v9 software. This matrix was used to perform moving-window analysis by plotting the correlation between hour x and hour x–24. This way, each data point in the graph is in itself a daily comparison. The $\Delta_{t(day)}$ values were calculated as the averages and standard deviations for the respective moving-window curve data points subtracted from the 100 % similarity value (Marzorati *et al.*, 2008).

Functional organization (*Fo*) is the result of the action of the microorganisms that are most fitting to the ongoing environmental-microbiological interactions. In order to graphically represent the structure of the bacterial community in rotifers Pareto-Lorenz evenness curves were set up (Marzorati *et al.*, 2008).

5.2.8. Sequencing of DNA from PCR fragments and bacterial isolates

DGGE bands were cut out with a sterile scalpel. Each fragment was washed with 200 µl of sterile MilliQ water and DNA eluted in 50 µl at 4 ºC for five days. Five microlitres of eluted DNA from each DGGE band was re-amplified using the same conditions described above. Purity of bands was checked by loading 100 ng of PCR product on a new DGGE as described above, using as control the same sample from which bands were excised. Those PCR products that yielded a single band migrating the same distance as the band in the original sample were then purified and sequenced.

DNA extracted from bacterial isolates was amplified by using the primers and conditions described in PCR-DGGE section and 100 ng of resultant PCR products were loaded in a DGGE as described above. DNA from strains showing a unique band in the corresponding profile were then amplified with primers 27f and 907Mr (Ampe *et al.*, 1999) (Table 5.1) as previously pointed out (Hoefel *et al.*, 2005) and resultant PCR products were used for sequencing.

Purification of PCR products was conducted by using ExoSap-It kit (GE Healthcare) following manufacturer's instructions. Sequencing reactions were prepared with the ABI Prism dRhodamine Terminator cycle sequencing ready reaction kit (GE Healthcare). The mixture of 90 – 200 ng of cleaned PCR product with 2 µl of Terminator mix, 6.4 pmol of the corresponding primer and distilled water up to 10 µl were added. The components were mixed and the tube loaded in the thermal cycler. The conditions of the sequencing reaction were one cycle at 94 ºC for 3 min and twenty-five cycles with 96 °C for 10 s, 50 °C for 5 s and 60 °C for 4 min. Extension products were purified using an ethanol/magnesium chloride precipitation procedure for the removal of the non-incorporated dye terminators. The pellet was dried at 30 °C in a centrifuge with a vacuum device and stored at -20 °C. Once extension products were purified, electrophoresis was carried out in an ABI PRISM™ 310 DNA Sequencer (Applied Biosystems). Prior to sample loading, pooled and dried reaction products were suspended in loading buffer (Applied Biosystems), containing five parts of deionized formamide to one part of 25 mM EDTA (pH 8.0).

The sequences obtained were compared against nucleotide sequences in the GenBank of the National Centre for Biotechnology Database (NCBI) using the Basic Local Alignment Search Tool (BLAST). The closest identified relatives were included in further phylogenetic analyses. Multiple sequence alignment of nucleotide sequences was generated by ClustalW (http://www.ebi.ac.uk) and a phylogenetic tree was constructed by the neighbour-joining approach with Jukes Cantor correction within MEGA v4.0 software (Tamura *et al.*, 2007). Robustness of tree topology was verified through calculating bootstrap values for the neighbour-joining tree and through comparison with the topology of a maximum likelihood tree, calculated using the default settings.

5.3. Results

5.3.1. Colonization and residence time of *Listonella anguillarum* 90-11-287 and *Vibrio splendidus* DMC-1 in rotifers

Real-time PCR analysis of samples taken at the end of colonization (t_0) revealed that rotifers were loaded with $2.1 \cdot 10^4$ bacteria·rotifer^{-1} of *L. anguillarum* (Treatment L) and $1.5 \cdot 10^3$ bacteria·rotifer^{-1} of *V. splendidus* (Treatment V) (Figure 5. 1).

Concentration of *L. anguillarum* in rotifers of Treatment L was 10^4 bacteria·rotifer^{-1} for 48 h, after being transferred to rearing tanks and maintained under normal larval rearing conditions. These values corresponded with CFU counts obtained from MA plates. Then, concentration of *L. anguillarum* decreased progressively until 10^2 bacteria·rotifer^{-1}, although counts on MA plates remained constant.

In treatment V, concentration of *V. splendidus* in rotifers decreased nearly two logarithmic units after 24 h, maintaining afterwards values below 10^2 bacteria·rotifer^{-1} and accounting less than 1 % of total bacteria until the end of the experiment.

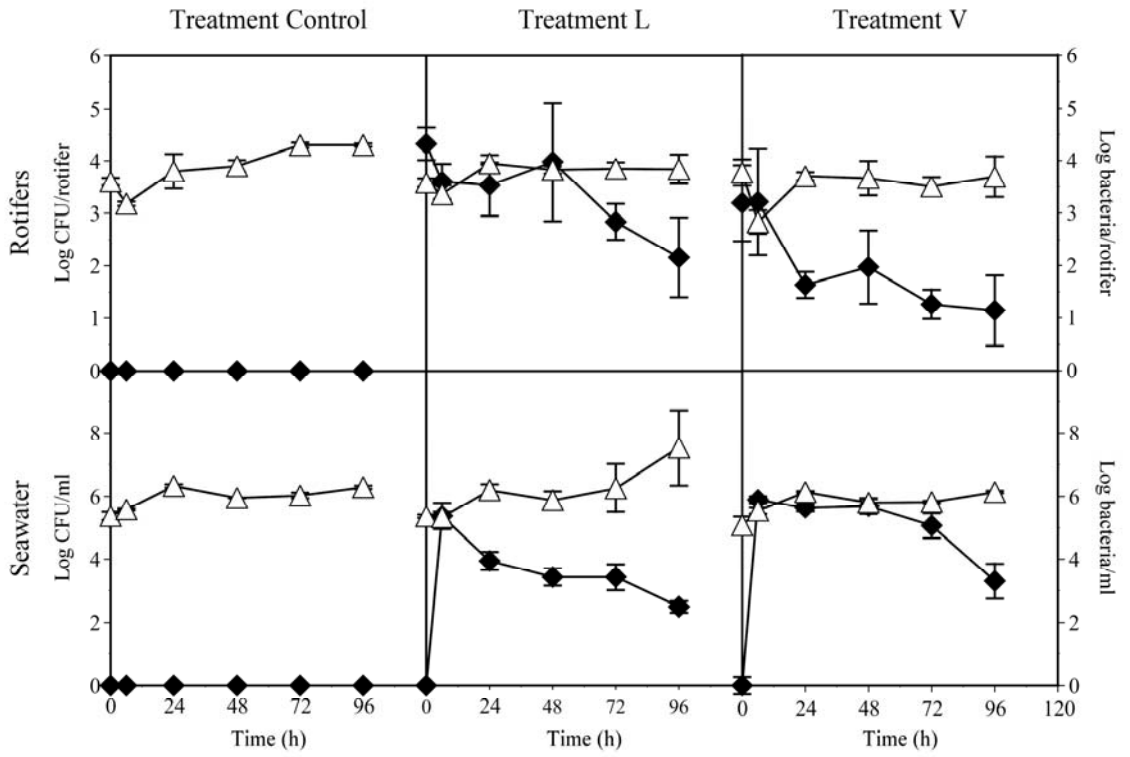

Figure 5.1. Residence time of *L. anguillarum* 90-11-287 (Treatment L) and *V. splendidus* DMC-1 (Treatment V) in the rotifer *Brachionus plicatilis* and transference rate to seawater of rearing tanks. Concentrations of total heterotrophic bacteria (Log CFU/ml) obtained from MA plate counts (Δ) and of target *Vibrionaceae* bacteria (Log bacteria/ml) obtained from real-time PCR analysis (♦) are represented. All data are mean and standard deviation of two independent assays and each assay value is the average of a technical duplicate.

Both pathogens were released from rotifers to seawater mainly during the first 6 h. Concentration of *V. splendidus* increased initially up to 10^6 bacteria·ml^{-1} (75 % of total viable cultivable bacteria) which was maintained for 48 h. *L. anguillarum* was released

to seawater in the first 6 h with a concentration of 10^5 bacteria·ml^{-1}, decreasing afterwards to 10^4 – 10^3 bacteria·ml^{-1} (< 1 % total viable cultivable bacteria).

At the beginning of the experiment (0 h), the total number of target bacteria in rotifers was $3.15 \cdot 10^{10}$ for *L. anguillarum* in Treatment L and $2.31 \cdot 10^7$ for *V. splendidus* in Treatment V. After 6 h, the total number of target bacteria in the seawater of tanks was $1.40 \cdot 10^{10}$ bacteria for *L. anguillarum* and $4.66 \cdot 10^{10}$ bacteria for *V. splendidus*. Therefore, total *L. anguillarum* in seawater can be explained by bacterial release from rotifers to seawater. Differently, total *V. splendidus* in seawater was three logarithmic units higher than the initial quantity in rotifers, which indicates not only bacterial release from rotifers to seawater but also the growth of pathogen in the seawater of tanks.

Neither *L. anguillarum* nor *V. splendidus* were detected by real-time PCR in rotifers or seawater from tanks of Treatment Control.

Despite rotifers survival was not affected after being colonized by the pathogens, a drop in rotifers concentration occurred (c60 % in Treatments L and V and c40 % in Treatment Control) in the first 24 h (Figure 5.2). A progressive recovery of initial rotifers densities was observed, especially in controls. Treatments with pathogens performed similarly.

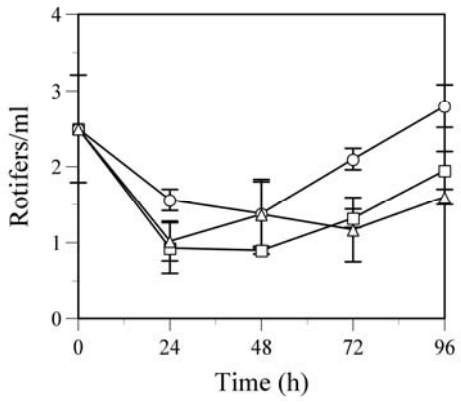

Figure 5.2. Concentration of rotifers control (○) and rotifers loaded with *Listonella anguillarum* 90-11-287 (□) or *Vibrio splendidus* DMC-1 (Δ) throughout maintenance in larvae rearing tanks. Data are mean and standard deviation of two independent assays.

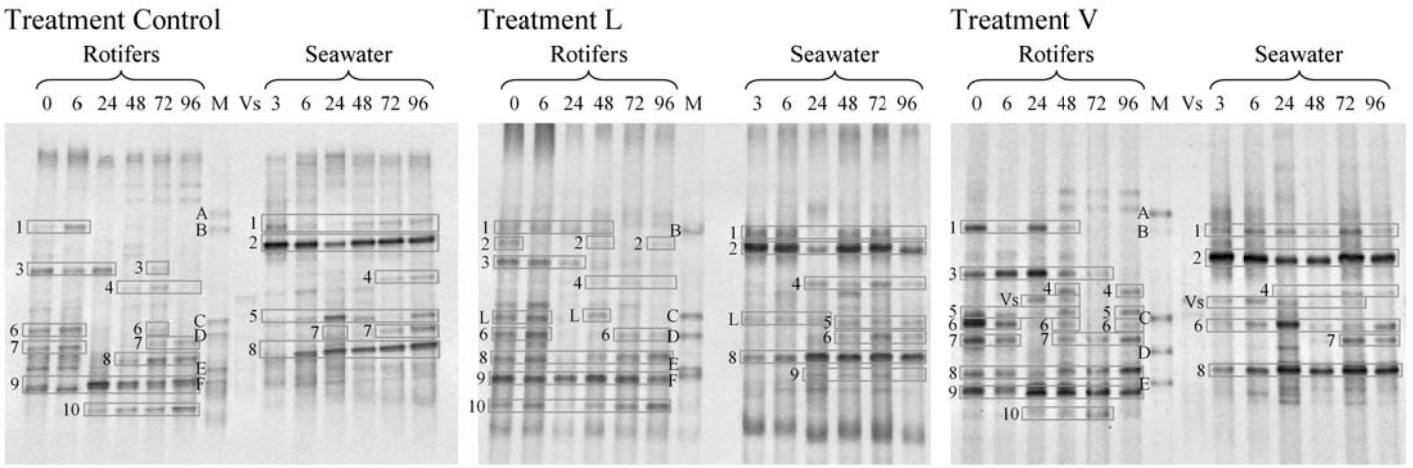

Figure 5.3. PCR-DGGE profile of 16S rDNA fragments of rotifers (*Brachionus plicatilis*) and seawater samples from Treatments Control, L and V. M: marker (A: *Kordia algicida*, B: *Tenacibaculum discolor*, C: *Listonella anguillarum* 90-11-287, D: *Phaeobacter* sp., E: *Ruegeria mobilis*, F: *Flexibacter* sp.), Vs: *V. splendidus* DMC-1 profile.

5.3.2. Effect of pathogens colonization on bacterial communities associated with rotifers

5.3.2.1. Plate counting and isolation of predominant strains

Total bacteria load in rotifers and seawater did not change throughout the experiment and was similar in all treatments (Figure 5.1). In rotifers, 10 – 20 % of total bacteria grown on MA also grew on TCBS. This proportion was maintained 48 h in rotifers of Treatment Control and in rotifers loaded with *V. splendidus* (Treatment V), whereas in rotifers from Treatment L this percentage was maintained for 72 h. In the seawater of all treatments, the highest proportion (3 %) of total viable cultivable bacteria grown on TCBS was detected just before partial renovation (48 h), being less than 1 % in the rest of times analysed (data not shown).

Eleven colonies showing different morphology on MA were isolated from rotifers and seawater throughout the experiment. Nine morphotypes could be purified and maintained through the consecutive plating on MA.

5.3.2.2. Analysis of DGGE profiles

DGGE profiles of the three treatments (Control, L and V) showed a similar number of bands (Figure 5.3). The introduced pathogenic strains were detectable in rotifers and seawater at different times. In Treatment L, rotifers showed a band (band L) with equal migration as *L. anguillarum* (band C of the marker) during the first 48 h, being also observed in seawater for 24 h. In Treatment V, a light band migrating the same distance as *V. splendidus* (band Vs) was detected at 24 – 48 h in rotifers, and for 72 h in seawater (Figure 5.3) of the same treatment. No band with migration corresponding to the introduced fish pathogens was detected in rotifers or seawater of Treatment Control (Figure 5.3).

The parameters range-weighted richness (*Rr*), dynamics (*Dy*) and functional organization (*Fo*) were calculated to analyse the influence of *L. anguillarum* 90-11-287 and *V. splendidus* DMC-1 in rotifers bacterial community.

Rr was always < 10 in Treatment Control, indicating that the diversity of this system is low (Marzorati *et al.*, 2008). The presence of *L. anguillarum* (Treatment L) or

V. splendidus (Treatment V) produced an increase of *Rr* in rotifers at the beginning of maintenance experiment and after 24 – 48 h in seawater, due to the apparition of the bands corresponding to the introduced pathogens. *Rr* values similar to Treatment Control were recorded at the end of the experiment, when bands corresponding to the introduced strains disappeared (Table 5.2).

Daily deviations in rotifers and seawater were established using a moving-window analysis (Figure 5.4). Rotifers of Treatment Control demonstrated daily shifts of 50 % in the first 24 h, 20 % at 48 h and about 30 % at the end of the experiment. Rotifers of Treatment L shifted between 15 % and 20 % during the first 48 h and changed up to 31 % at 72 h and 11 % at 96 h. Rotifers of Treatment V showed shifts of up to 23 % in the first 48 h and changes of up to 17 % in the last two days.

Table 5.2. Range-weighted richness parameter of rotifers and seawater throughout maintenance experiment.

Treatment	Time (h)	Rotifers		Seawater	
		Rr	[a] Rank	*Rr*	Rank
Control	0	7.20	L	2.40	L
	6	7.20	L	3.75	L
	24	1.80	L	3.75	L
	72	9.30	L	5.40	L
	96	5.00	L	5.40	L
L	0	16.20	M	2.40	L
	6	12.80	M	2.40	L
	24	3.20	L	9.80	L
	72	7.20	L	12.80	M
	96	7.20	L	9.80	L
V	0	9.80	L	5.00	L
	6	12.80	M	7.20	L
	24	7.20	L	20.00	M
	72	7.35	L	16.20	M
	96	9.60	L	7.20	L

[a] L: Low microbial diversity (*Rr* < 10); M: Intermediate microbial diversity (30 > *Rr* >10)

Seawater of Treatment Control showed daily shifts of 35 % in the first 24 h, changing then gradually over time (daily changes of up to 19 %). Treatment L demonstrated daily shifts in seawater of 50 – 44 % in the first 48 h and 14 – 19 % at the end of the experiment. Treatment V seawater community shifts were not so pronounced as those from control and L treatments, as demonstrated by the shorter fluctuations in the moving-window analysis plot (up to 80 % correlation and thus 20 % change).

Average values for these rates of change were calculated and expressed as rates of change [$\Delta_{t(day)}$]. The total community changed more rapidly in rotifers of Treatment Control (31.99 ± 8.97 %) and in seawater of Treatment L (31.75 ± 17.90 %) than in rotifers and seawater from the other treatments (15 – 19 %).

In order to graphically represent the structure of rotifers bacterial community, Pareto-Lorenz evenness curves were constructed based on DGGE profiles (Figure 5.5). Over time, it was observed that 20 % of the bands present in DGGE profiles of rotifers and seawater of the three treatments corresponded with 25 – 35 % (on average 30 %) of the cumulative band intensities.

5.3.2.3. Identification of bands in DGGE profiles and MA cultivable bacteria by sequencing

Table 5.3 and Figure 5.6 show the relationship of sequences from the excised DGGE bands and MA isolates to other sequences in GenBank. Bands with the same number in DGGE profiles showed similar sequences (Figure 5.3).

A band related to the plastidial DNA of the microalgae *I. galbana* (band 2), added to the seawater of rearing tanks was detected in all seawater samples.

Alpha Proteobacteria were dominant in rotifers and seawater. Band 8 was related to *Ruegeria gelatinovorans* and identified in rotifers and seawater of all treatments, being more predominant in seawater and with increasing intensity throughout the experiment. Band 9, related to *Ruegeria* sp, was only present in rotifers and showed similar intensity in the three treatments and at all times analysed.

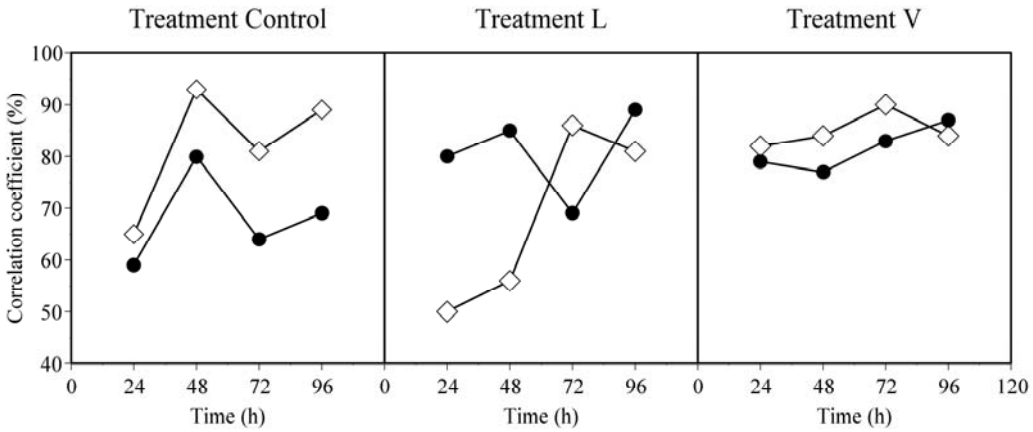

Figure 5.4. Moving-window analysis based on DGGE profiles of rotifers (●) and seawater (◊) of Treatment Control, Treatment L and Treatment V. Each data point in the graph is in itself a daily comparison, as it represents the correlation between the samples of hour x and hour x–24.

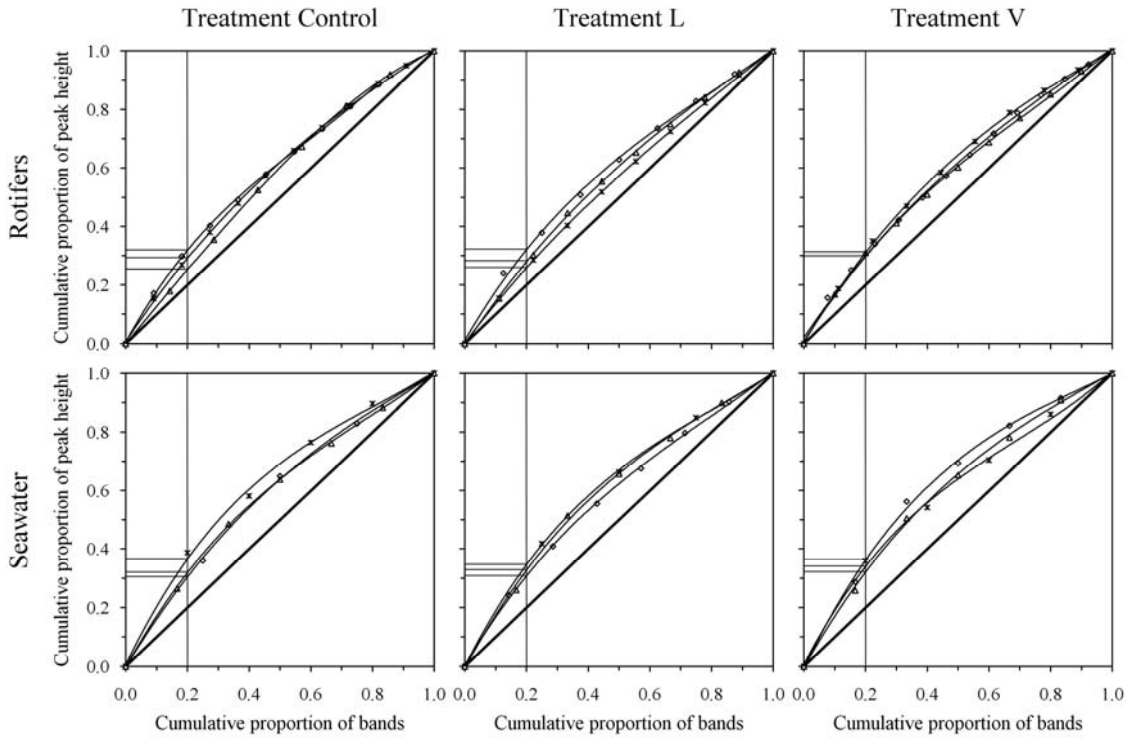

Figure 5.5. Pareto-Lorenz distribution curves based on PCR-DGGE analysis on t_0 (*), t_{48} (◊) and t_{96} (Δ) of rotifers and seawater of Treatment Control, Treatment L and Treatment V. The dashed vertical line at the 0.2x axis level is plotted to evaluate the range of the Pareto values.

Bands identified as bacteria of γ-Proteobacteria group showed different patterns depending on treatment. Two bands related to uncultured γ-Proteobacteria (bands 5 and 6) were detected in rotifers and seawater of all treatments.

Band 5 (Uncultured *Oceanospirillaceae* bacterium) was detected in the seawater of Treatments Control and L but not in the seawater of Treatment V, being only detected in the rotifers loaded with *V. splendidus*. Band 6 (Uncultured γ-Proteobacterium) was detected in rotifers from all treatments at early (0 – 6 h) and late (72 – 96 h) stages. In seawater, band 6 was only detected in Treatments L and V and at different times.

Table 5.3. Similarities to other sequences in GenBank of excised DGGE bands and of bacterial isolates taken from rotifers and seawater samples.

Band/Isolate	Origin	Closest relative			
		% Similarity	Species	Accession number	Taxon
L	Rotifers / Seawater	98 (102/104)	*Listonella anguillarum*	AY069970	γ-Proteobacteria
Vs	Rotifers / Seawater	99 (158/160)	*Vibrio splendidus*	AY227706	γ-Proteobacteria
1	Rotifers / Seawater	95 (111/116)	Bacteroidetes bacterium	AM709742	CFB group
2	Rotifers / Seawater	95 (134/141)	*Isochrysis galbana* plastid	X75518	Microalgae
3	Rotifers	93 (138/148)	*Tenacibaculum soleae*	AM989479	CFB group
4	Rotifers / Seawater	92 (76/82)	*Polaribacter dokdonensis*	DQ004686	CFB group
5	Rotifers / Seawater	91 (76/83)	Uncultured *Oceanospirillaceae* bacterium	DQ421459	γ-Proteobacteria
6	Rotifers / Seawater	98 (110/111)	Uncultured γ-Proteobacterium	AJ810628	γ-Proteobacteria
7	Rotifers / Seawater	91 (133/146)	Uncultured *Marinobacter* sp	AY494608	γ-Proteobacteria
8	Rotifers / Seawater	96 (123/128)	*Ruegeria gelatinovorans*	AJ295988	α-Proteobacteria
9	Rotifers / Seawater	95 (141/148)	*Ruegeria* sp	AB274753	α-Proteobacteria
CVR1	Rotifers	96 (734/764)	*Tenacibaculum soleae*	AM989479	CFB group
CVR2	Rotifers	92 (658/708)	Uncultured *Pseudoalteromonas* sp	AM941176	γ-Proteobacteria
CVR3	Rotifers / Seawater	96 (717/746)	*Polaribacter dokdonensis*	DQ004686	CFB group
CVR4	Rotifers	98 (673/684)	*Microbacterium* sp	EF204413	Actinobacteria
CVR5	Rotifers	98 (670/683)	*Alteromonas alvinellae*	FJ040190	γ-Proteobacteria
CVR6	Rotifers	96 (668/690)	*Ruegeria mobilis*	AB255401	α-Proteobacteria
CVR7	Rotifers	98 (678/687)	*Roseivirga spongicola*	DQ080996	CFB group
CVR8	Rotifers	97 (670/686)	*Ruegeria mobilis*	AB255401	α-Proteobacteria
CVR9	Rotifers	94 (472/501)	Uncultured marine bacterium	FM211095	γ-Proteobacteria

Sequences were aligned to their closest relatives by using the BLAST 2.2.15.

In Treatment L, band 6 appeared at the end of the experiment in seawater and, in Treatment V, it was detected at all times analysed. Band 7 (Uncultured *Marinobacter* sp) was detected in rotifers of Treatments Control and V at the beginning and at the end of the experiment, being only detectable in the seawater in the last 48 h.

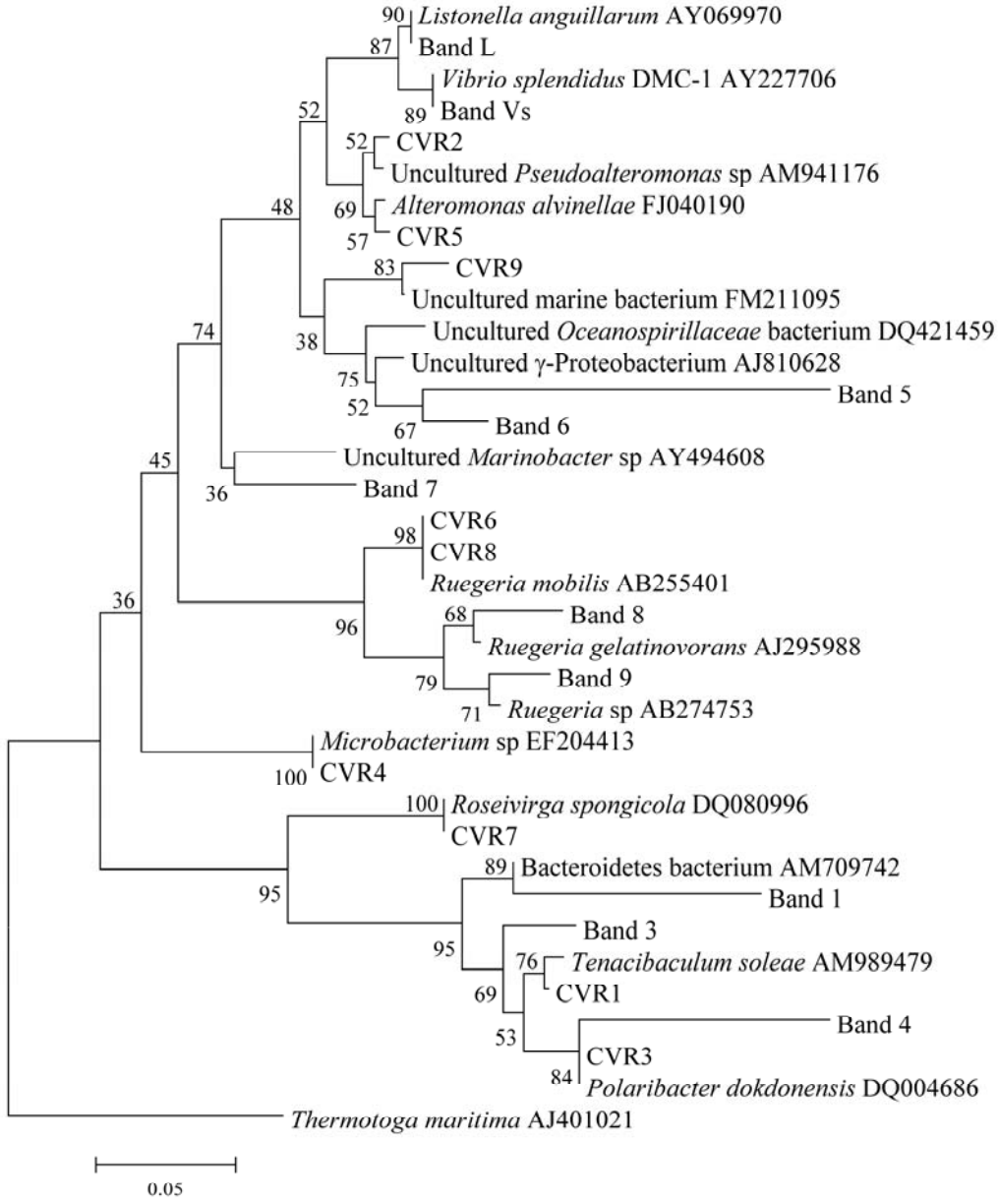

Figure 5.6. Phylogenetic dendrogram constructed with partial (about 150 bp) 16S rDNA sequences showing the relationship of DGGE bands and MA isolates to different bacterial groups. The tree was constructed with the neighbour-joining method of the MEGA program package and the Jukes-Cantor correction. *Thermogota maritima* was used as the out-group.

A band related to a Bacteroidetes bacterium (band 1) was detected in the seawater of the three treatments at all times analysed and was also detected until 6 h in rotifers used as control, and until 48 h in the rotifers loaded with *L. anguillarum* or *V. splendidus*. Two other bands (bands 3 and 4), related to bacteria from CFB (*Cytophaga-Flexibacter-Bacteroides*) group, were also detected in all treatments. Band 3 (*Tenacibaculum soleae*) was only associated with rotifers at the initial period, from 0 to 24 – 72 h, and band 4 (*Polaribacter dokdonensis*) was detected in both rotifers and seawater, at the end of the maintenance experiment.

Bacterial isolates from MA plates were genotyped by DGGE and characterized by sequencing. Ten of the isolates showed unique genotypes, with a single band migration pattern. Three isolates (CVR2, CVR5 and CVR9) were identified as members of the γ-Proteobacteria group, three morphotypes (CVR1, CVR3 and CVR7) were related to the CFB group, two strains (CVR6 and CVR8) were identified as *Ruegeria mobilis* (α-Proteobacteria) and one (CVR4) as *Microbacterium* sp (*Actinobacteria*). All isolates were plated on TCBS to check their ability to grow in this medium. Isolates CVR2 (*Pseudoalteromonas* sp) and CVR4 (*Microbacterium* sp) grew in TCBS.

DGGE bands 3 and 4 showed a clear phylogenetic similarity to isolates CVR1 (*Tenacibaculum soleae*) and CVR3 (*Polaribacter dokdonensis*), respectively (Figure 5.6). The other isolates were not detected in DGGE. Bands 8 and 9 were clearly clustered with species of the genus *Ruegeria* but not with the isolates CVR6 and CVR8.

5.4. Discussion

L. anguillarum 90-11-287 and *V. splendidus* DMC-1 are pathogenic strains that caused turbot larvae mortality when introduced via rotifers in challenge trials (Planas *et al.*, 2005; Thomson *et al.*, 2005). The present study demonstrates that both strains have different capabilities to colonize and maintain in the rotifer *Brachionus plicatilis*.

Colonization of rotifers by *L. anguillarum* was ten-fold higher than by *V. splendidus*, even though the concentration in the bacterial suspension used for rotifers infection was ten-fold lower for *L. anguillarum* than for *V. splendidus*. Different colonization levels have also been reported for several *Vibrio* species bioencapsulated in *Artemia franciscana* nauplii (Gómez-Gil *et al.*, 1998). Maintenance of infected rotifers under larval rearing conditions conducted to an initial release of bacteria to seawater. *V.*

splendidus attained higher concentrations and grew in the seawater of tanks whereas *L. anguillarum* maintained in rotifers longer and did not grow in the seawater.

The different capability of *L. anguillarum* and *V. splendidus* to colonize and maintain in rotifers or seawater could be explained by their natural occurrence in different niches. *L. anguillarum* appears commonly associated with rotifers (Mizuki *et al.*, 2006; Verdonck *et al.*, 1997), whereas *V. splendidus* is considered the dominant species of vibrio-plankton (Le Roux and Austin, 2006). *V. splendidus* has been mainly isolated from *Artemia* but usually not found in rotifers (Reid *et al.*, 2009; Thomson *et al.*, 2005). The better adaptation of *V. splendidus* to the planktonic environment (Le Roux and Austin, 2006) could explain its capability to grow in seawater of the rearing tanks. Other factors such as temperature and available organic matter might also determine growth of vibrios (Sugita *et al.*, 2008).

In the present study, *L. anguillarum* and *V. splendidus* were detected in rotifers and seawater at least four days, without any additional input of organic matter. In standard larval rearings, rotifers are added daily to rearing tanks and excretion products of larvae and prey are released to the surrounding seawater. This would enhance the proliferation and maintenance of the pathogens in the system (rotifers and/or seawater), promoting the re-infection of larvae (Olsson *et al.*, 1998; Sugita *et al.*, 2008).

The influence of *L. anguillarum* 90-11-287 and *V. splendidus* DMC-1 in rotifers bacterial community was analysed by DGGE. The incorporation of *L. anguillarum* or *V. splendidus* did not displace or modify significantly the bacterial microbiota present on rotifers not reducing bacterial diversity, as demonstrated by *Rr* values, which increased temporary due to the presence of the pathogens.

Daily shifts in rotifer bacterial community of Treatment Control can be explained by the bacterial exchange between rotifers and seawater. Moving-window analysis of Treatment L and Treatment V showed differences with respect to control. In Treatment L changes in rotifers were marked by the disappearance of *L. anguillarum* band in rotifers between 48 and 72 h. Less marked changes were observed in Treatment V, showing a higher change in seawater between 72 and 96 h, when the band corresponding to *V splendidus* disappeared.

Over time, it was observed that 20 % of the bands corresponded with an average of 30 % of the cumulative intensity of the bands in the three treatments (Control, L and

V) which implies that rotifers bacterial community did not present a marked species dominance. This fact may facilitate the rapid temporary colonization of rotifers and the subsequent release to seawater of opportunistic bacteria, such as *Vibrionaceae*, which have high growth rates and are potentially harmful to fish larvae (Skjermo and Vadstein, 1993). Similar results have been obtained for three different *B. plicatilis* strains cultured under different feed types, regimes and mixtures of three probiotic strains (Qi *et al.*, 2009). The authors did not observed clear correlations between bacterial community composition and the introduction of the different probionts.

In the present study, bacterial community in rotifers and seawater was composed by groups previously found in hatcheries of different fish species: α-Proteobacteria, γ-Proteobacteria and CFB group (Griffiths *et al.*, 2001; McIntosh *et al.*, 2008; Sandaa *et al.*, 2003; Schulze *et al.*, 2006). Only two DGGE bands corresponded to bacteria isolated from MA plates, suggesting that bacterial groups present in rotifers may not be easily cultivable. On the other hand, most MA isolates were not detected in DGGE profiles, which would mean that they might not be predominant but better cultivable. Colonies of α-Proteobacteria (*Ruegeria* spp), CFB (*Polaribacter dokdonensis*, *Roseivirga spongicola*) and the *Actinobacteria* species were clearly distinguished on MA plates. However, γ-Proteobacteria colonies were morphologically very similar, making difficult to distinguish between different morphotypes. On the other hand, the use of selective media may also have limitations to identify bacteria. TCBS medium is described as specific for *Vibrionaceae* bacteria but, in this study, it has been observed that strains related to other bacterial groups, including Gram negative (*Pseudoalteromonas* sp) and Gram positive bacteria (*Microbacterium* sp) were able to grow in this medium. Similarly, other authors have found that some Gram positive and Gram negative bacteria different to *Vibrionaceae* grow in TCBS medium (López-Torres and Lizárraga-Partida, 2001).

All these results suggest that the analysis of bacterial communities should be based on both culture dependent and culture independent techniques, as previously pointed out by other authors (McIntosh *et al.*, 2008; Schulze *et al.*, 2006).

One of the isolates in MA, identified as *Microbacterium* sp, a Gram positive bacterium belonging to the *Anctinobacteria* class, was detected in all treatments but only in rotifers. *Microbacterium* sp has been previously found associated with *Artemia franciscana* cysts (Orozco-Medina *et al.*, 2002), seawater, cultured algae, shellfish, finfish (Schulze *et al.*, 2006) and cod (Reid *et al.*, 2009) but it has never been related to

rotifers before. The origin of the *Microbacterium* sp strain isolated in this study could be the microalgae *I. galbana* (Makridis *et al.*, 2006).

Two strains related to *Ruegeria* genus, belonging to the *Roseobacter* clade (α-Proteobacteria), became predominant in rotifers when the introduced *Vibrionaceae* or other strains related to *Tenacibaculum* genus disappeared. The success of bacteria from the *Roseobacter* clade over other bacterial groups (Griffiths *et al.*, 2001; Hjelm *et al.*, 2004a, b; Sandaa *et al.*, 2003), due to the production of antagonistic compounds (Brinkhoff *et al.*, 2004; Bruhn *et al.*, 2005; Ruíz-Ponte *et al.*, 1999), could explain the predominance and persistence of *Ruegeria* spp strains in rotifers observed in the present study.

Understanding the ecology of *Vibrionaceae* bacteria in rotifer cultures is important to comprehend the mode fish larvae get infected and to develop effective prevention strategies. In this study, the residence time for *L. anguillarum* 90-11-287 and *V. splendidus* DMC-1 in the system was long enough to potentially infect fish larvae, by ingestion of prey and/or by active intake or contact with surrounding seawater.

The higher colonization and residence time of *L. anguillarum* in rotifers and the higher transfer level from rotifers to seawater of *V. splendidus* could determine different infection patterns in both pathogens. Accordingly, infection by *L. anguillarum* would mainly occur by ingestion of rotifers and subsequent colonization of the gut, whereas the infection with *V. splendidus* would be favoured by an active intake of seawater or by direct contact with surrounding seawater, colonizing preferably the gut or the epidermis, respectively. In agreement with this hypothesis, Grisez *et al.* (1996) found that *L. anguillarum*, previously incorporated in *Artemia* nauplii, infected turbot larvae through digestive tract. However, in a challenge carried out with turbot larvae fed on rotifers infected with *L. anguillarum* 90-11-287, Planas *et al.* (2005) observed that the pathogen did not colonize the intestine but affected the epidermis of larvae whereas *V. splendidus* DMC-1 has been detected as the main species in the gut of diseased turbot and cod larvae (Reid *et al.*, 2009; Thomson *et al.*, 2005).

A different adhesion to mucus of both species could explain these findings. The capability of *Vibrionaceae* bacteria to adhere to different types of mucus in different fishes is strain dependent (Balebona *et al.*, 1995; Bordas *et al.*, 1998). Olsson *et al.* (1996) showed that *L. anguillarum* did not adhere specifically to turbot intestinal mucus but accumulated close to intestinal mucus interfaces and subsequently penetrated them.

Therefore, knowledge on the adhesion to mucus capabilities would be determinant to understand the infection patterns for *L. anguillarum* 90-11-287 and *V. splendidus* DMC-1, which is essential in the prevention of infections and in the design of control strategies (e.g. probiotics).

This is the first study that evaluates specifically by real-time PCR the colonization and residence time of pathogenic strains in rotifers maintained under fish larval rearing conditions. In this chapter it has been demonstrated that *Listonella anguillarum* 90-11-287 and *Vibrio splendidus* DMC-1, when introduced via rotifers in the larvae rearing tanks, were able to remain in rotifers and seawater enough time to infect fish larvae. The behaviour of both strains was different as colonization and residence time of *V. splendidus* in the rotifer *Brachionus plicatilis* were lower than that of *L. anguillarum*, passing faster to the seawater.

The bacterial community of rotifers did not present marked species dominance. The incorporation of *L. anguillarum* or *V. splendidus* did not reduce bacterial diversity and shifts could be explained by bacterial exchange between rotifers and seawater.

5.5. References

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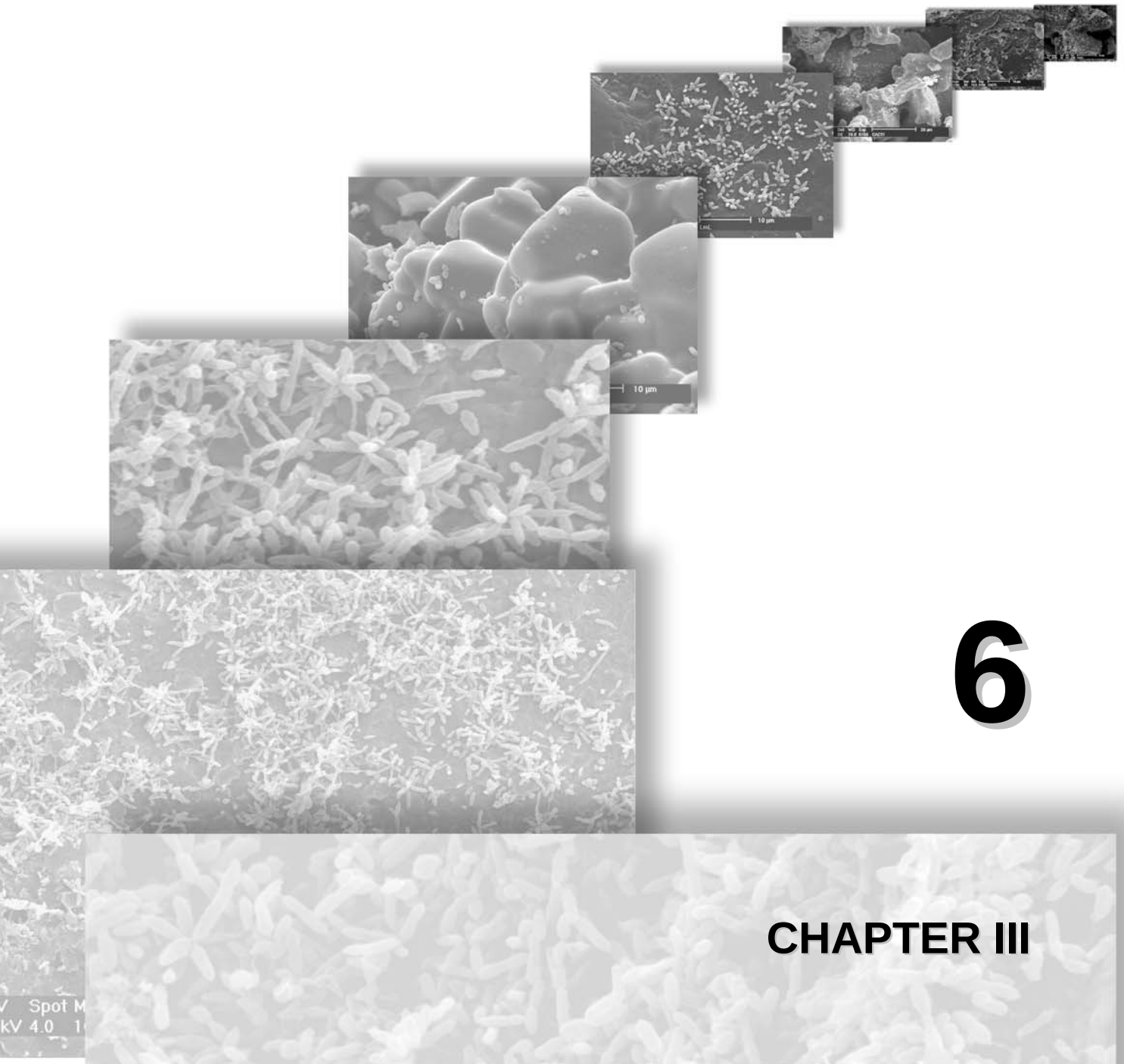

6

CHAPTER III

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Application of biofilters with the probiotic strain *Phaeobacter* 27-4 against *Vibrionaceae* infections in the rearing of turbot (*Psetta maxima*) larvae

6.1. Introduction

Intensive aquaculture production of marine fish larvae has been hampered by the loss of stock through disease and to low and unpredictable survivals. Turbot larvae are reared at high densities and exposed to high bacterial levels at first feeding (Olafsen, 2001), providing an environment suitable for growth of opportunistic bacteria (Skjermo *et al.*, 1997) which may lead to reduced growth and survival of larvae (Salvesen *et al.*, 1999). Turbot larval mortality at first-feeding on rotifers and the subsequent transition to *Artemia* nauplii or metanauplii has contributed to important losses in turbot farms (Ringø and Birbeck, 1999).

Mortalities caused by bacterial infections may be reduced or eliminated by antibiotics but leading to the emergence of antibiotic resistant bacteria (Cabello, 2006). Alternatively, other approaches for bacterial control have been proposed, such as disinfection of fish eggs (Salvesen and Vadstein, 1995), production of rotifer axenic cultures (Dhert *et al.*, 2001; Douillet, 1998) and treatment of live prey for partial decontamination (Giménez *et al.*, 2006; Munro *et al.*, 1993, 1999). However, some of these alternatives are difficult to implement at industrial scale. On the other hand, the loss of a stable microbial population (*K* strategists) is a common consequence of disinfection, leading to a rapid colonization of the rearing systems by opportunistic bacteria (*r* strategists), which are potentially harmful to fish larvae (Skjermo and Vadstein, 1993). Probiotics are defined by FAO/WHO as “live microorganisms which, when administered in adequate amounts, confer a health benefit on the host”. The use of probiotics constitutes a strategy for improvement of fish larvae viability and avoids the use of antibiotics and disinfectants (Gatesoupe, 1999; Irianto and Austin, 2002; Planas *et al.*, 2006; Vine *et al.*, 2006).

The *Roseobacter gallaeciensis* cluster (*Roseobacter* clade – α-Proteobacteria) (Brinkhoff *et al.*, 2008) includes several *Phaeobacter* strains with demonstrated antagonistic activity against different bacterial groups by production of thiotropocin and/or tropodithietic acid (Brinkhoff *et al.*, 2004; Grossart *et al.*, 2004; Hjelm *et al.*, 2004a). It has been demonstrated a beneficial effect of *Phaeobacter* spp on larvae of fish (Planas *et al.*, 2006) or shellfish (Balcázar *et al.*, 2007; Ruíz-Ponte *et al.*, 1999) by the addition of bacterial culture suspensions (Balcázar *et al.*, 2007; Planas *et al.*, 2006) or cell extracts (Ruíz-Ponte *et al.*, 1999) to the larvae rearing tanks.

Phaeobacter strain 27-4, isolated from a turbot farm (Hjelm *et al.*, 2004a, b), antagonizes the turbot larvae pathogens *Listonella anguillarum* and *Vibrio splendidus* (Hjelm *et al.*, 2004a, b) due to the production of tropodithietic acid, which is related to the production of a dark brown pigment (Bruhn *et al.*, 2005). Planas *et al.* (2006) demonstrated the *in vivo* probiotic effect of *Phaeobacter* 27-4 using a delivery protocol to turbot larvae based on the bioencapsulation of the probiotic bacteria in rotifers. This protocol increased the survival of larvae challenged with *L. anguillarum*, a pathogen that causes external infections in turbot larvae (Planas *et al.*, 2005). However, *Phaeobacter* 27-4 did not colonize turbot larvae and continuous additions (each 48-72 h) of the probiont were necessary to maintain an effective concentration of the probiotic in seawater and rotifers (Planas *et al.*, 2006).

Marine *Roseobacter* clade members are globally distributed (Buchan *et al.*, 2005; Wagner-Döbler and Biebl, 2006) being able to rapidly colonize a variety of inorganic and organic marine surfaces, including marine algae and dinoflagellates (Dang and Lovell, 2002; Mayali *et al.*, 2008). Roseobacters associated with particles have shown a higher antimicrobial activity than free-living members (Long and Azam, 2001). In accordance with this Roseobacters feature, the antagonistic activity of *Phaeobacter* 27-4, which is able to colonize inert surfaces (Bruhn *et al.*, 2006), has been associated with biofilm formation in the air-liquid interface (Bruhn *et al.*, 2005, 2007).

The intestinal microbial community in aquatic organisms cannot be considered independently of the microbiota of fish environment (seawater). Accordingly, Verschuere *et al.* (2000) defined probiotics in aquaculture as "a live microbial adjunct which has a beneficial effect on the host by modifying the host-associated or ambient microbial community, by ensuring improved use of the feed or enhancing its nutritional value, by enhancing the host response towards disease, or by improving the quality of its ambient environment", extending the concept to bacteria that produce a beneficial

effect on the host by modification of seawater microbial community. This new broadened definition would also include microorganisms present in the seawater or in the rearing environment but not colonizing the host (intestinal tract, gills or skin). Therefore, this probiotic concept opens to microorganisms which improve seawater quality (Dalmin *et al.*, 2001), or act as biocontrol agents reducing the level of pathogenic (Kennedy *et al.*, 1998) or total bacteria (Makridis *et al.*, 2001) in seawater.

Biofilters colonized with slow-growing (*K* strategist) bacteria with biofilm forming capability have been used for production of matured seawater, resulting in enhancement of survival in halibut yolk-sac larvae (Skjermo *et al.*, 1997) and growth of turbot larvae (Salvesen *et al.*, 1999) by improvement of microbial seawater quality. Based on the capability of *Phaeobacter* 27-4 to attach to surfaces and to form biofilms, biofilters prepared with supports previously inoculated and colonized with probionts could be used as a strategy to deliver and maintain probiotic bacteria in the rearing systems. The application of probiotic strains, as *Phaeobacter* 27-4, in biofilters instead of repeated additions (e.g. bioencapsulated in rotifers) would improve the permanence of the probiont in larval rearing systems and could be a useful tool for the control of pathogens and opportunistic bacteria in industrial units.

The aim of this study was to develop an application protocol of *Phaeobacter* 27-4 based on the immobilization of the probiotic bacteria in biofilters. In this chapter it has been analysed the growth and biofilm formation of *Phaeobacter* 27-4 on different supports (plastic balls, sintered glass pellets and ceramic cylinders) and culture conditions (soaked stagnant and submerged with or without agitation), determined the permanence of *Phaeobacter* 27-4 in supports when maintained under turbot rearing conditions and evaluated the *in vitro* antagonistic activity of *Phaeobacter* 27-4 biofilters against *Listonella anguillarum* 90-11-287 and *Vibrio splendidus* DMC-1, as well as the antagonism against *L. anguillarum* 90-11-287 under larval rearing conditions. The *in vivo* probiotic effect of matured biofilters of *Phaeobacter* 27-4, prepared with the selected support, was demonstrated at pilot-scale on turbot larvae challenged with *L. anguillarum* 90-11-287 via infected rotifers.

6.2. Materials and methods

6.2.1. Bacterial strains, culture media and supports

The fish pathogens *Listonella anguillarum* 90-11-287 (Skov *et al.*, 1995) and *Vibrio splendidus* DMC-1 (Hjelm *et al.*, 2004a; Thomson *et al.*, 2005), and the fish probiotic *Phaeobacter* strain 27-4 (Hjelm *et al.*, 2004a) were kindly provided by Prof. Lone Gram (DTUAqua, Denmark).

All the strains were kept at -80 °C in Marine Broth (MB, Difco 2219) with glycerol (final concentration: 15 %) and routinely cultured on MB as previously described (Prol *et al.*, 2009). Counts of *L. anguillarum* and *V. splendidus* were carried out on Tryptic Soy Agar (TSA, Cultimed) during experiments in microcosms. Thiosulfate Citrate Bile Sucrose (TCBS) agar (Cultimed) was employed for total *Vibrionaceae* counts when indicated.

Three commercial aquaculture biofilter supports made from different materials (plastic, sintered glass and ceramics) were selected for growth of *Phaeobacter* 27-4. Table 6.1 shows characteristics of the supports employed.

Table 6.1. Characteristics of supports employed for growing *Phaeobacter* strain 27-4

Support	Commercial name	Material	Volume (cm^3)	Liquid retention (% v/v)	Surface:Volume ratio ($m^2 \cdot l^{-1}$)
Plastic balls (PB)	Filter balls (JBL)	Plastic	6.37	23.55	0.46
Sintered glass pellets (GP)	Micro Mec (JBL)	Glass	1.23	40.65	$1.5 \cdot 10^3$
Ceramic cylinders (CC)	Bio Max	Ceramics	2.51	31.68	$4.6 \cdot 10^3$

6.2.2. Extraction of *Phaeobacter* 27-4 bacteria from the supports

Four methods were assayed, independently or combined, to recover the probiotic bacteria from the biofilter. A factorial experiment was carried out by means of a complete first order orthogonal design with replicates in each point, according to the procedures described by Box *et al.* (1978). The following treatments were applied:

washing (W), homogenization (H), sonication (two cycles of 30s, S) and addition of Tween 80 (5 $mg \cdot l^{-1}$, T). Ten plastic balls (PB) were introduced in 80 ml of a culture suspension containing 10^7 $CFU \cdot ml^{-1}$ of *Phaeobacter* 27-4 and carefully agitated. Fifty sintered glass pellets (GP) or twenty ceramic cylinders (CC) were introduced in 100 ml of a *Phaeobacter* 27-4 suspension (10^7 $CFU \cdot ml^{-1}$) and gently mixed. Soaked supports were transferred to a sterile flask and incubated at 20 ºC in the dark. Samples were taken at 0 h and after 48 h for microbiological analysis. The criterion for model acceptance was based on Student's t test ($p < 0.05$) for the coefficients, and on Fisher's F test ($p < 0.05$) applied to the ratios [experimental error/total error] and [lack of fit/experimental error], for the total validity.

6.2.3. Culture of *Phaeobacter* 27-4 on different supports and conditions

Phaeobacter 27-4 was reactivated in 4.5 ml of MB by incubation at 20 ºC for 72 h in the dark and stagnant conditions. One millilitre of the obtained pre-culture was used to inoculate 100 ml of MB (10^7 $UFC \cdot ml^{-1}$) which were employed to inoculate the different supports with *Phaeobacter* 27-4. The number of supports used for preparation of biofilters was ten PB, fifty GP or twenty CC and corresponded to 50 – 60 % (v/v) of the total volume in the flasks. All cultures were conducted in duplicate, at 20 ºC and in the dark.

Three culture conditions were assayed for each biofilter support:

i) *Soaked stagnant* (SkS) cultures were prepared by introduction of the supports units in the *Phaeobacter* 27-4 suspension and gently mixed. Then, the soaked supports were transferred to a sterile flask and incubated under stagnant conditions.

ii) *Submerged stagnant* (SbS) cultures were prepared by introduction of the supports units in the suspension of *Phaeobacter* 27-4. Incubation took place under stagnant conditions.

iii) *Submerged agitated* (SbA) cultures were prepared by introduction of the supports units in *Phaeobacter* 27-4 suspension. Incubation was carried out under agitated conditions (100 rpm).

Cultures of *Phaeobacter* 27-4 (10^7 CFU·ml^{-1}) with no added supports were used as controls.

Samples from the supports were taken at different times from 0 to 120 h. Recovery of bacteria from the supports was conducted using the method selected in the extraction experiment (see above). One unit of each support was taken under sterile conditions and washed twice with 50 ml of autoclaved seawater, applying a gentle agitation to eliminate non adhered bacteria. PB were subsequently vortexed in 10 ml of autoclaved seawater to separate the attached bacteria. GP and CC were homogenized in a sterile mortar with 10 ml of autoclaved seawater and the homogenate transferred to a sterile tube. Microbiological analysis was conducted in the resultant bacterial suspensions and in the homogenates.

6.2.4. *In vitro* inactivation and inhibition of *Listonella anguillarum* 90-11-287 and *Vibrio splendidus* DMC-1 by *Phaeobacter* 27-4 biofilters

Phaeobacter 27-4 was grown for four days on each type of support according to the culture conditions detailed above. One PB, five GP or two CC were washed twice with 50 ml of autoclaved seawater, applying a gentle agitation, and introduced in bacterial suspensions of *L. anguillarum* 90-11-287 or *V. splendidus* DMC-1.

For the inactivation experiment, pathogenic bacteria were re-suspended in 100 ml of autoclaved seawater, at a concentration of 10^4 – 10^5 CFU·ml^{-1}. For the inhibition experiment, pathogenic bacteria were suspended in 100 ml of MB at a concentration of 10^2 – 10^3 CFU·ml^{-1}. Suspensions of *L. anguillarum* and *V. splendidus* in autoclaved seawater or MB, in which autoclaved units of each support were introduced, were used as controls. All experiments were run in duplicate.

Samples from bacterial suspensions were taken for microbiological analysis at different times (0 – 120 h). Samples from the supports were taken at the beginning (0 h) and at the end of the experiment (120 h).

6.2.5. Residence time of *Phaeobacter* 27-4 in biofilters maintained under turbot larval rearing conditions

Phaeobacter 27-4 was grown on the different supports for four days in the conditions that resulted in the formation of rosette-shaped microcolonies and/or a biofilm. The supports were collected and introduced in a plastic mesh to pack the different *Phaeobacter* biofilters containing forty PB, two-hundred GP or one-hundred CC. The resulting volume of each biofilter corresponded with 250 ml (0.5 % v/v of total volume in 50 l tanks). Each biofilter was washed twice with 5 l of sterile seawater, introduced in tanks with 50 l of green seawater (seawater containing $2 \cdot 10^5$ cells·ml^{-1} of the microalgae *Isochrysis galbana*) and matured at 18 ºC under turbot larval rearing conditions, with continuous aeration (> 90% oxygen saturation) and illumination for eleven days (264 h). The light intensity at seawater surface was 3.5 µE·sec^{-1}·m^{-2} (day light was provided by fluorescent lamps). A tank with no added bacteria was used as control. All maturation experiments were conducted in duplicate.

Samples from supports and seawater were taken at different times (0 – 264 h) for microbiological analysis and DNA extraction.

6.2.6. Effect of matured *Phaeobacter* 27-4 biofilters against *Listonella anguillarum* 90-11-287 in tanks maintained under rearing conditions

The strain *L. anguillarum* 90-11-287 previously used to validate *Phaeobacter* 27-4 probiotic effect (Planas *et al.*, 2006), was chosen as target in antagonism trials carried out in non-axenic rearing conditions.

Antagonistic activity of *Phaeobacter* 27-4 immobilized on the CC against *L. anguillarum* 90-11-287, was tested in tanks with 2.5 l of green seawater (low nutrients level) or green seawater with 5 ml of MB (high nutrients level) under turbot larval rearing conditions as described above. The purpose of MB addition was to reproduce the high organic matter conditions present in rearing tanks, as consequence of live feed, faeces and larvae debris.

Phaeobacter 27-4 (10^7 CFU·ml^{-1}) was cultured on CC for four days in SbS conditions at 20 °C. Biofilters composed by one-hundred CC with the probiotic were prepared and matured for ten days (240 h) in green seawater ($2 \cdot 10^5$ *I. galbana* cells·ml^{-1})

under the conditions described above. Matured biofilter was washed twice with 5 l of autoclaved seawater and distributed in smaller units, with twenty cylinders each, which were introduced into 2.5 l green seawater tanks. Twenty-five microlitres of a 24 h culture of *L. anguillarum* were used to inoculate the tanks (10^4 – 10^5 CFU·ml^{-1}). Treatments without any bacteria added (*Control*), with *L. anguillarum* (*La*), with *Phaeobacter* 27-4 biofilter (*BPh*) and with both the pathogen and the probiotic biofilter (*LaBPh*) were performed with and without addition of 5 ml of MB. In *Control* and *La* tanks, a biofilter with autoclaved CC was introduced. All treatments were run in duplicate.

Samples from seawater were taken at different times from 0 to 144 h for microbiological cultures (total bacteria and total *Vibrionaceae*) and real-time PCR analyses. Samples from the supports were taken at the beginning (0 h) and at the end of the experiment (144 h) and treated as stated above.

6.2.7. *In vivo* probiotic effect of *Phaeobacter* 27-4 biofilters on turbot larvae

Newly hatched larvae (day zero) of turbot were kindly provided by Insuíña Pescanova (Mougás, Southern Coast of Galicia, Spain). One day after hatching, larvae were acclimatized by raising progressively the temperature from 15 to 18 ºC during the following three days. The larvae transferred at day two to 5 l tanks filled with 4 l of green seawater (35 larvae l^{-1}), and maintained in the dark. The seawater of tanks was moderately aerated (> 90 % oxygen saturation) and at day three, light intensity at the surface was adjusted to 3.5 µE·sec^{-1}·m^{-2} (day light provided by fluorescent lamps).

Turbot larvae were fed daily on rotifers enriched with *I. galbana* from day three until day ten after hatching. Density of rotifers was adjusted daily (3 – 5 rotifers·ml^{-1}) and the seawater of the rearing tanks was partially (30 – 40 %) renewed every two days from first feeding with the subsequent addition of 0.25 l of *I. galbana* culture ($2·10^7$ cells·ml^{-1}).

To ascertain the probiotic effect of *Phaeobacter* 27-4 biofilters, turbot larvae were challenged with *L. anguillarum* 90-11-287 using an infection model, based on the bioencapsulation of the pathogen in the rotifer *Brachionus plicatilis*, described by Planas *et al.* (2005). Briefly, one million of rotifers enriched with *I. galbana* were

filtered over a nylon mesh of 30 μm pore size and re-suspended in 1 l of 1-μm-filtered seawater. Two-hundred millilitres of a 24 h culture of *L. anguillarum* (10^9 CFU·ml^{-1}) were incorporated in rotifer cultures and the volume adjusted to 5 l with 1-μm-filtered seawater. After 3 h, rotifers were filtered over a nylon mesh of 30 μm pore size, washed and added to the larvae. Challenged larvae were fed with rotifers loaded with *L. anguillarum* 90-11-287 at days four, six and eight post-hatching.

Phaeobacter 27-4 biofilters using ceramic supports were prepared and matured in green seawater as described above. After maturation, biofilters with twenty cylinders were introduced in a 250 μm nylon mesh to avoid turbot larvae getting into the biofilters. Four different treatments were performed:

i) *Control*: larvae fed on rotifers enriched with *I. galbana*.

ii) *BPh*: larvae fed on rotifers enriched with *I. galbana* and in presence of *Phaeobacter* 27-4 biofilter.

iii) *La*: larvae fed on alternate days with *I. galbana* enriched rotifers loaded with *L. anguillarum* and without *Phaeobacter* 27-4 biofilter.

iv) *LaBPh*: larvae fed on alternate days with *I. galbana* enriched rotifers loaded with *L. anguillarum* and in presence of *Phaeobacter* 27-4 biofilter.

In *Control* and *La* tanks, a biofilter prepared with autoclaved CC was introduced. All treatments were conducted in duplicate.

The bottom of the tanks was siphoned daily to remove and count dead larvae. The significance of the probiotic effect was ascertained using the t test for related samples (5 % level of significance) to compare accumulated mortalities in *Control* versus experimental groups along the trial.

Samples from larvae, rotifers and seawater were taken at different times (day three – day ten after larval hatching) for microbiological cultures (total bacteria and total *Vibrionaceae*) and DNA extraction. Samples from the supports were taken at the beginning (day three post-hatching) and at the end of the experiment (day ten post-hatching).

6.2.8. Microbiological methods

Ten-fold serial dilutions were prepared in autoclaved seawater from samples of 30-μm-filtered seawater, from bacterial suspensions obtained from the washing of the plastic supports, from homogenized porous supports (GP and CC) or from homogenized samples of four-hundred rotifers or ten larvae. One hundred microlitres of each dilution were plated on Marine Agar (MA, Difco 2219), incubated for 120 h at 20 ºC and in the dark for total bacteria quantification.

Phaeobacter 27-4 colonies were identified on MA plates by their dark brown pigmentation. When indicated, MA plates with 30 – 300 colonies were replicated on TCBS agar for total *Vibrionaceae* counts and incubated at 20 ºC and in the dark for 24 – 48 h. In inactivation and inhibition experiments, *L. anguillarum* and *V. splendidus* were quantified on TSA plates after incubation for 24 – 48 h at 20 °C and in the dark.

6.2.9. Pigment quantification

One unit of each support was introduced in a volume of autoclaved seawater (10 ml for PB and 5 ml for GP and CC) and shaken vigorously. A volume of 1.5 ml from the resultant solution was centrifuged (13,000 × g / 10 min / 20 ºC), filtered over 0.2 μm and the optical density at 398 nm (OD_{398}) measured in the bacteria free supernatant. In submerged cultures, pigment production was also quantified in the bacteria free supernatant of the culture.

Pigment adsorption by the different materials was checked by introduction of two PB, ten GP or four CC in 20 ml of a bacteria free supernatant obtained from a four days culture of *Phaeobacter* 27-4, followed by the incubation in the dark at 20 ºC for 24 h. Bacteria free supernatant of *Phaeobacter* culture was used as control. After the incubation period, one unit of each support was treated as stated above and the washing solutions used for OD_{398} analysis.

6.2.10. Scanning Electron Microscopy (SEM)

One unit of each support, previously washed twice with 50 ml of autoclaved seawater and gently agitated to eliminate non adhered bacteria, was fixed in 2 %

glutaraldehyde (TAAB Essentials for Microscopy) prepared in sodium cacodylate (Electron Microscopy Sciences) buffer (0.1 M, pH 7.4) at 4 ºC for 2 h and washed with 0.1 M sodium cacodylate buffer (30 min / three times). Samples were then dehydrated by washings with 30, 50, 70, 80 and 95 % ethanol for 30 min each and two washings of 30 min with absolute ethanol. Ethanol was then gradually substituted by amylacetate and samples exposed to 73 atm and 31.3 ºC until totally dehydrated. Finally, each sample was set under a carbon sheet and sprinkled with gold (10 – 20 mm) prior to be analysed under a scanning electron microscopy Philips XL30.

6.2.11. DNA extraction

Ten-thousand rotifers and ten larvae were treated as outlined by Prol *et al.* (2009) and the resultant homogenates were frozen at -20 ºC until DNA extraction. Seawater samples (50 ml) were filtered over a nylon mesh with 30 μm pore size and centrifuged (5,000 × g / 10 min / 20 ºC). The resultant pellet was washed once with autoclaved seawater (1.5 ml) and frozen (-20 ºC) until use.

DNA was extracted from samples of larvae, rotifers, seawater and solutions obtained from the washing and homogenates of supports (1 ml) by a Phenol: Chloroform: Isoamyl Alcohol protocol as previously described (Prol *et al.*, 2009). All DNAs were frozen (-20 ºC) until analysed.

6.2.12. Real-time PCR

Phaeobacter 27-4 and *L. anguillarum* 90-11-287 were specifically quantified by real-time PCR (Prol *et al.*, 2009) during the trials carried out in non-axenic conditions, to avoid the interference of the other bacteria present in the tanks.

Quantification of DNA extracted from duplicate samples was by running a real-time PCR, also in duplicate, with the adequate standard curves and set of primers. The primers VA (5'-CATACGCAGCCAAAAATCAA-3'; 5'-GCACTGTCCGTCATGCTATC-3') and tdbR (5'-GCGCTTCTCAAGCACCTAAC-3'; 5'-ACGGTGTCCCTTACCTTCCT-3') were selected for real-time PCR analysis. These primers were designed within genes encoding for virulence or antagonism and

have shown to be specific for *L. anguillarum* 90-11-287 and *Phaeobacter* 27-4 (Prol *et al.*, 2009).

The standard curves were prepared in presence of the appropriate background organism (larvae, rotifers or microalgae). One-hundred nanograms of ten-fold (larvae and rotifers) or one-hundred-fold (seawater) diluted DNA were mixed with the adequate primers (final concentration 4.3 μM) and the *Power* SYBR® Green master mix (Applied Biosystems), containing the AmpliTaq Gold® DNA polymerase, the double strand DNA-binding dye *Power* SYBR® Green and the reference dye ROX®. The real-time PCR program was performed on a 7500 Fast Real-Time PCR System (Applied Biosystems). PCR amplification was performed as described in Prol *et al.* (2009). Dissociation curves were used for testing specificity of the PCR products. No template DNA controls (NTC) and samples of the appropriate background matrix without any bacteria added were used as negative controls in each run.

6.2.13. PCR-DGGE

Purified DNA was amplified with primers gc338f (5'-CGCCCGCCGCGCGCG GCGGGCGGGGCGGGGGCACGGGGGGACTCCTACGGGAGGCAGCAG-3') and 518r (5'- ATTACCGCGGCTGCTGG-3') spanning the V3 region of the 16S rDNA (Muyzer *et al.*, 1993). Amplification was performed in a GeneAmp 2700 PCR System (Applied Biosystems) thermal cycler. One-hundred nanograms of DNA were mixed with each primer (0.25 μM), the deoxynucleotides triphosphate mix (dNTPs) (0.2 mM), $MgCl_2$ (1.5 mM), PCR buffer for *Taq* polymerase (1X), *Taq* polymerase (0.05 $U \cdot \mu l^{-1}$) and BSA (0.4 mM) in a final volume of 50 μl.

PCR consisted in a total of thirty one cycles. Activation of *Taq* polymerase was by one cycle at 94°C for 5 min followed by twenty cycles with three steps (94ºC for 1 min, 55ºC for 1 min, 72ºC for 3 min). A "touchdown" PCR was performed to increase amplification specificity and to reduce the formation of spurious by-products (Muyzer *et al.*, 1993). The annealing temperature was set 10 °C above the expected annealing temperature (55°C) and decreased by 0.5 °C every second step of the twenty cycles, followed by ten additional cycles (94 °C for 1 min, 55 °C for 1 min and 72 °C for 3 min). A final extension was performed at 72 °C for 10 min. The amplification products were analysed first by electrophoresis in 2 % agarose gels and quantified by using the Hyperladder IV (Bio-Rad) ladder and Quantity One software (Bio-Rad).

Five hundred nanograms of the PCR products were analysed by DGGE in a Bio-Rad DCode following the procedure described by Muyzer *et al.* (1993). Samples were loaded on 8 % (w/v) polyacrylamide gels in 1X TAE. All parallel electrophoreses were performed at 60 °C using gels containing a 30 to 60 % gradient UF (100 % corresponded to 7 M urea and 40 % [v/v] formamide) increasing in the direction of electrophoresis. Gels were run at 20 V for 10 min, followed by 3 h at 200 V and stained by bathing for 30 min in a 0.5 % (v/v) ethidium bromide solution and rinsed for 30 min in distilled water.

6.2.14. Analysis of DGGE profiles

The obtained DGGE profiles were subsequently processed using Quantity One v4.4.1 software package (Bio-Rad) and the parameters range-weighted richness (*Rr*), dynamics (*Dy*) and functional organization (*Fo*) (Marzorati *et al.*, 2008) were calculated.

Range-weighted richness (*Rr*) relates the number of bands present in the DGGE profile to the percentage of denaturing gradient needed to describe the total diversity of the sample analysed. This index was calculated during biofilters maturation for both biofilters and seawater samples. In the challenge trial with turbot larvae, *Rr* was calculated at all times larvae and seawater were analysed (from day three until day ten after hatching of larvae) to determine the influence of introduced strains in the carrying capacity of the system.

Dynamics of the microbial community (*Dy*) can be interpreted as the number of species that on average come to significant dominance (above the detection limit of the technique) at a given habitat during a defined time interval. Dynamics can be calculated using the comparison tool moving-window analysis. A matrix of similarities for the densiometric curves of the bands patterns was calculated based on the Pearson product-moment correlation coefficients employing STATISTICA v9 software. This matrix was used to perform moving-window analysis by plotting the correlation between two dates.

Functional organization (*Fo*) is the result of the action of the microorganisms that are most fitting to the ongoing environmental-microbiological interactions. In order to graphically represent the structure of the bacterial community in turbot larvae Pareto-Lorenz evenness curves were set up (Marzorati *et al.*, 2008).

A matrix was constructed from DGGE profiles of rotifers, larvae and seawater samples taken during the challenge trial, considering the presence or absence of the individual bands and the relative contribution of each band (by percentage) to the total intensity of each sample. This matrix was used to calculate the distance matrix using normalized Euclidean distances (root mean square differences) with the software STATISTICA v9. Finally, a dendrogram comparing the different samples was obtained by using the unweighted-pair group method using average linkages and STATISTICA.

6.3. Results

6.3.1. Culture of *Phaeobacter* 27-4 on different supports and conditions

The capability of *Phaeobacter* 27-4 to grow, to produce pigment and to form a biofilm was evaluated by culturing the probiont on commercial supports used in aquaculture (PB, GP and CC) and under three different culture conditions (SkS, SbS and SbA).

The results of the factorial experiment carried out to select a procedure for extraction of *Phaeobacter* bacteria from the different supports (Table 6.2) showed that homogenization (H) did not affect negatively to CFU counts and that the effect of sonication (S) and the addition of Tween 80 (T) was not significant. Consequently, homogenization of the supports, without sonication or the addition of Tween 80, was chosen as the most adequate extraction method. The concentration of bacteria extracted from supports at t_0 was similar to the concentration of the bacterial suspension used to inoculate them (10^7 CFU·ml^{-1}). Hence, the extraction method did not affect the viability of the bacteria (Table 6.2).

Phaeobacter 27-4 was able to grow on all supports and under all conditions tested. In porous supports (GP and CC), the highest concentration of *Phaeobacter* was observed in SkS and SbA cultures whereas in PB cultures, *Phaeobacter* showed the highest level when cultured in SbA conditions (Figure 6.1).

Culture conditions influenced greater plastic than porous supports. Stable concentrations of bacteria were observed after 24 – 48 h on the three supports, with exception of PB-SbA, where *Phaeobacter* concentration decreased markedly from 96 h.

Table 6.2. Equations coefficients obtained from the factorial design applied to test homogenization (H), sonication (S) and addition of Tween 80 (T) as treatments for the extraction of *Phaeobacter* 27-4 bacteria from PB, GP and CC ($Y = a_0 + a_1H + a_2S + a_3T + a_4HS + a_5HT + a_6ST + a_7HST$).

Supports	Time (h)	Coefficients								
		a_0	a_1	a_2	a_3	a_4	a_5	a_6	a_7	r^2
PB	[a]0	7.85	NA	NS	0.10	NA	NA	-0.03	NA	0.910
	48	10.46	NA	-0.34	-0.32	NA	NA	NS	NA	0.687
GP	0	6.91	0.10	-0.08	NS	0.06	NS	NS	NS	0.790
	48	9.93	0.36	NS	NS	NS	NS	NS	NS	0.433
CC	0	6.93	NS	NS	0.13	-0.17	NS	NS	NS	0.705
	48	9.76	NS	NS	-0.32	NS	NS	NS	NS	0.255

[a] *Phaeobacter* 27-4 bacteria extracted from the support at 0 h and 48 h (Log CFU·ml^{-1}). Initial concentration of *Phaeobacter* (Log CFU·ml^{-1}) = 7.5. NA: not applicable, NS: not significant

In PB, the concentration of *Phaeobacter* in the stationary phase was 10^7 CFU·cm^{-3} in SkS cultures, 10^8 – 10^9 CFU·cm^{-3} in SbS cultures and 10^9 – 10^{11} CFU·cm^{-3} when cultured in SbA conditions. In GP and CC, the concentration of *Phaeobacter* was 10^9 CFU·cm^{-3} in SkS cultures and 10^{10} – 10^{11} CFU·cm^{-3} in SbS and SbA. The highest concentration of *Phaeobacter* 27-4 was registered in GP-SkS and GP-SbA. Controls, without support, reached a concentration of 10^{10} CFU·ml^{-1} after 24 h.

SEM analysis showed that the attachment of bacteria was also influenced by the type of support and culture conditions. In SkS conditions, attachment to GP and CC was low, although the growth (CFU·cm^3) was two logarithmic units higher than for PB in the same conditions. In PB, a higher attachment and some rosette-shaped microcolonies were observed (Figure 6.1). In submerged conditions, the differences in attachment between supports were lower (Figure 6.1). For PB, which floated on the MB, agitation favoured attachment of the bacteria to the support. No significant differences were observed in GP between agitated and stagnant conditions. Isolated rosettes were detected on stagnant conditions on PB and GP. A well developed biofilm with rosette-shaped microcolonies was only observed on CC-SbS (Figure 6.1). No rosettes were observed in SbA conditions in any of the supports.

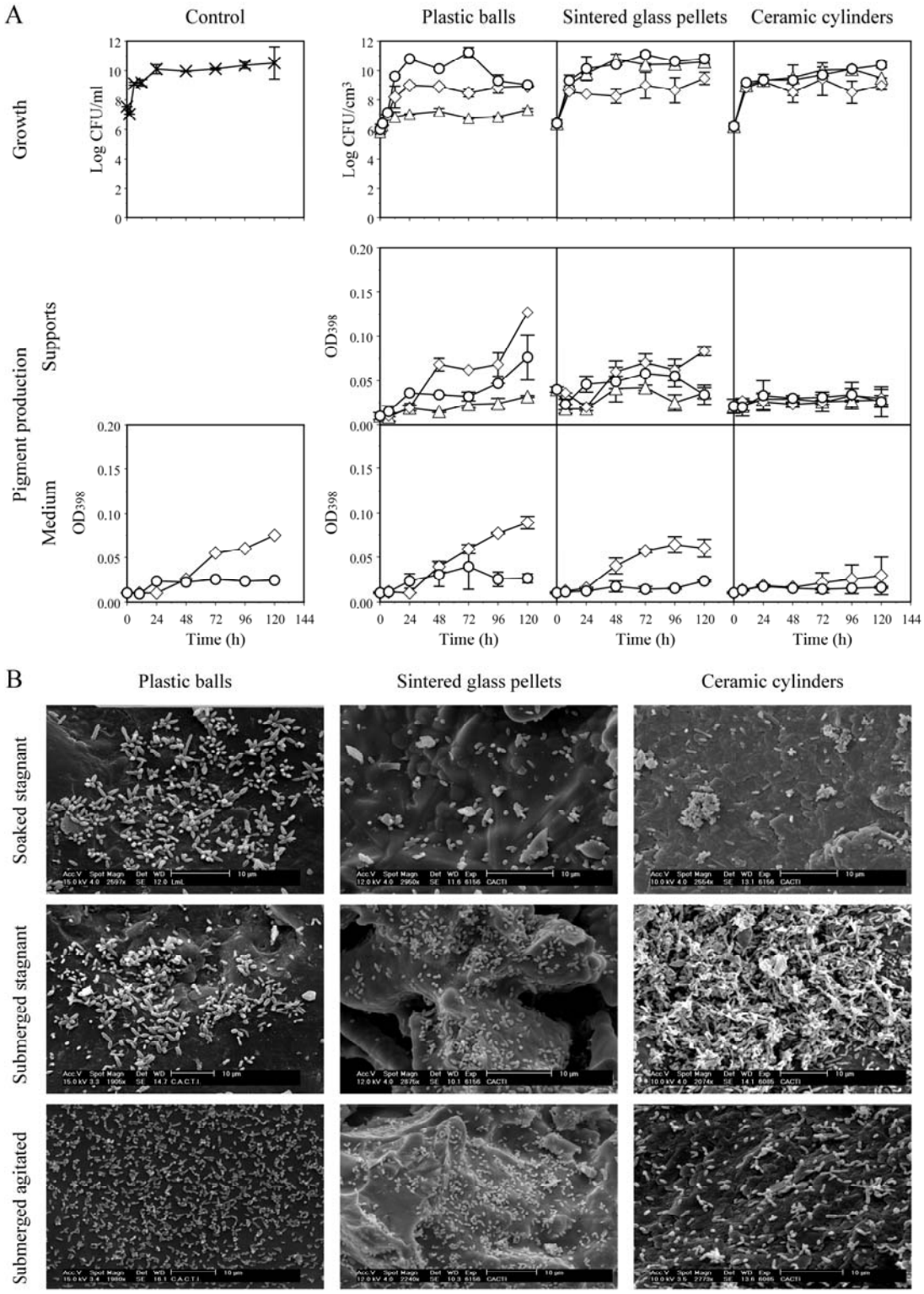

Figure 6.1. (A) Growth ($CFU \cdot cm^{-3}$) and pigment production (OD_{398}) by *Phaeobacter* 27-4 on different supports cultured under SkS (Δ), SbS (◊) or SbA (○) conditions. Growing ($CFU \cdot ml^{-1}$) of the control with no support added (×) as well as its pigment production (OD_{398}) are also shown. Data are mean and standard deviations of two independent assays. (B) SEM micrographs taken at 120 h of *Phaeobacter* 27-4 biofilters.

Pigment production was observed from 48 h in cultures of *Phaeobacter* 27-4 on PB and GP, showing in bacteria-free supernatants OD_{398} values similar to control. The highest OD_{398} was reached on SbS cultures, both in washing solutions from supports and in culture supernatants. A low pigment production was observed in GP and CC (Figure 6.1) which could not be explained by supports adsorption (data not shown).

6.3.2. *In vitro* inactivation and inhibition of *Listonella anguillarum* 90-11-287 and *Vibrio splendidus* DMC-1 by *Phaeobacter* 27-4 biofilters

The influence of different supports and culture conditions on the antagonistic activity of *Phaeobacter* 27-4 was studied in *L. anguillarum* or *V. splendidus* suspensions prepared in autoclaved seawater (inactivation test) or in MB (inhibition of growth test). The quantity of the introduced *Phaeobacter* strain varied depending on supports and conditions used (Table 6.3).

6.3.2.1. Inactivation

Initial *L. anguillarum* and *V. splendidus* concentrations were $10^3 – 10^4$ CFU·ml^{-1}. In controls, both pathogens grew up to 10^5 CFU·ml^{-1} in the first 48 h, maintaining this level until the end of the experiment (Figure 6.2).

For *L. anguillarum*, the presence of the biofilter with *Phaeobacter* 27-4 grown on all types of supports and conditions promoted a decrease in the concentration of the pathogen in one or two logarithmic units with respect to controls (Figure 6.2). In all cases, inactivation occurred from the first 24 h except for cultures on CC-SbS conditions and PB-SkS conditions, which produced the inactivation after 48 h. Inactivation was always maintained until the end of the experiment (120 h).

For *V. splendidus*, a decrease in one or two logarithmic units with respect to controls was observed when incubated with *Phaeobacter* biofilters prepared with any type of supports and culture conditions, with exception of SbS cultures with GP where no effect was observed (Figure 6.2). Inactivation times were different depending on the supports (Figure 6.2): 24 h with GP or CC and 48 h with PB.

Table 6.3. Concentration of *Phaeobacter* 27-4, *Listonella anguillarum* 90-11-287 e *Vibrio splendidus* DMC-1 on plastic, sintered glass and ceramic supports, throughout *in vitro* inactivation and inhibition experiments. Data are mean and standard deviation of two independent assays.

Support	Culture conditions	CFU·cm^{-3}						
		Inactivation				Inhibition		
		Phaeobacter		*L. anguillarum*	*V. splendidus*	*Phaeobacter*	*L. anguillarum*	*V. splendidus*
		[a] t_0	t_{120}			t_{120}		
PB	SkS	6.50 ± 0.10	5.63 ± 0.20	2.50 ± 0.52	2.31 ± 0.60	5.72 ± 0.36	2.52 ± 0.00	4.28 ± 0.44
	SbS	6.66 ± 0.78	5.67 ± 0.18	2.76 ± 0.27	2.89 ± 0.03	5.81 ± 0.33	2.67 ± 0.50	4.59 ± 0.32
	SbA	6.85 ± 0.45	5.64 ± 0.08	2.67 ± 1.72	2.92 ± 0.07	5.43 ± 0.72	2.47 ± 0.12	3.93 ± 0.35
	[b] Control	NA	NA	2.79 ± 0,50	2.87 ± 0.25	NA	3.79 ± 0.50	4.87 ± 0.25
GP	SkS	8.99 ± 0.00	7.99 ± 0.05	2.58 ± 1.76	4.71 ± 0.12	8.68 ± 0.77	2.51 ± 0.09	6.39 ± 0.22
	SbS	7.09 ± 0.38	7.84 ± 0.80	2.65 ± 0.60	4.39 ± 0.98	7.68 ± 0.37	1.91 ± 0.15	6.21 ± 0.05
	SbA	9.88 ± 0.94	8.56 ± 0.57	2.91 ± 0.19	3.91 ± 0.35	9.18 ± 1.11	2.01 ± 0.80	6.36 ± 0.06
	Control	NA	NA	4.97 ± 0.08	5.48 ± 0.23	NA	6.97 ± 0.08	7.48 ± 0.23
CC	SkS	7.90 ± 0.75	7.73 ± 0.22	4.23 ± 0.47	5.20 ± 0.08	9.03 ± 0.49	5.71 ± 0.86	5.80 ± 0.00
	SbS	7.31 ± 0.00	7.79 ± 0.14	5.12 ± 0.22	5.05 ± 0.01	8.89 ± 0.40	5.60 ± 0.35	6.20 ± 0.43
	SbA	8.05 ± 0.00	8.03 ± 0.16	4.40 ± 0.79	5.25 ± 0.04	9.03 ± 0.04	5.60 ± 0.00	6.20 ± 0.01
	Control	NA	NA	6.08 ± 0.26	6.13 ± 0.84	NA	7.08 ± 0.26	7.48 ± 0.23

[a] t_0: concentration of *Phaeobacter* 27-4 inoculated in the suspensions of fish pathogens; t_{120}: concentration of *Phaeobacter* 27-4 on supports at the end of the experiment. [b] Control: Culture of *L. anguillarum* or *V. splendidus* without *Phaeobacter* 27-4 biofilters. NA: Not analysed

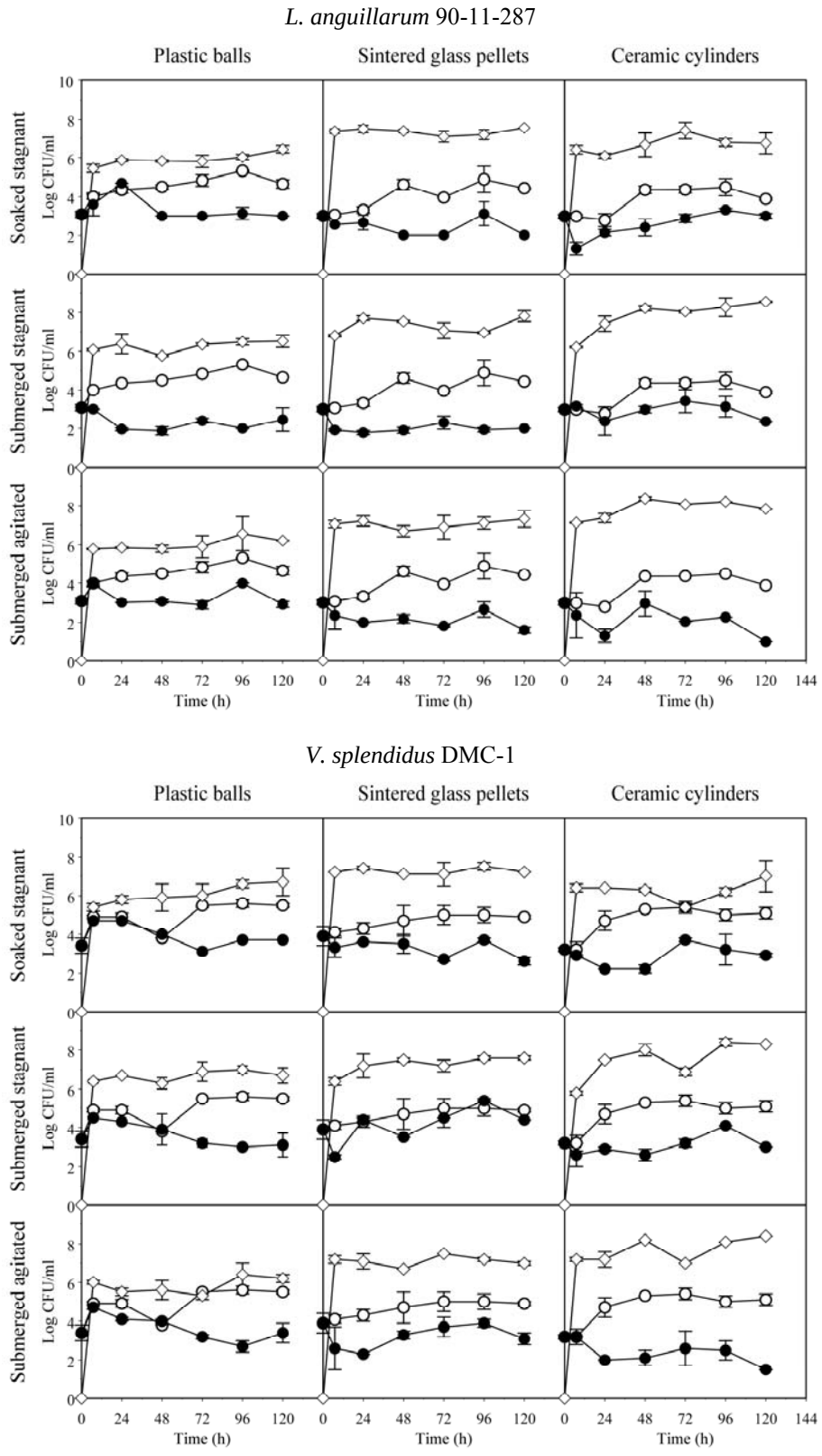

Figure 6.2. Inactivation of *Listonella anguillarum* 90-11-287 and *Vibrio splendidus* DMC-1 by *Phaeobacter* 27-4 immobilized on different supports. Pathogens concentration in presence (●) and absence (○) of *Phaeobacter*, as well as levels of *Phaeobacter* (◊) in the seawater are shown. Data are mean and standard deviations of two independent assays.

In the biofilters, *Phaeobacter* maintained the same concentration on CC throughout the experiment, independently of the culture conditions applied (Table 6.3). In PB and GP, *Phaeobacter* concentration diminished one logarithmic unit at the end of the experiment, except for GP-SbS cultures, in which the concentration increased in almost one logarithmic unit. *Phaeobacter* was released from all supports to seawater in the first 7 h, reaching concentrations of 10^6 – 10^7 CFU·ml^{-1}, which were subsequently maintained during all the experiment (Figure 6.2).

At the end of the experiment (120 h) both *L. anguillarum* and *V. splendidus* were detected in all supports (Table 6.3). In controls, the concentration for both pathogens in CC (10^6 CFU cm^{-3}) and GP (10^4 CFU cm^{-3}) was higher than in PB (10^3 CFU cm^{-3}). The presence of *Phaeobacter* 27-4 did not affect *L. anguillarum* and *V. splendidus* concentration attached to PB (Table 6.3) but reduced in one or two logarithmic units the concentration of both pathogens on the porous supports.

6.3.2.2. Inhibition

Initial *L. anguillarum* and *V. splendidus* concentrations were 10^2 CFU·ml^{-1}. Controls showed different growth on MB depending on the pathogen. *L. anguillarum* grew up to 10^7 CFU·ml^{-1} whereas *V. splendidus* reached values of 10^8 – 10^9 CFU·ml^{-1} in the first 24 h, which were maintained until the end of the experiment (Figure 6.3). The presence of *Phaeobacter* biofilter caused a higher inhibition for *L. anguillarum* than for *V. splendidus*.

For *L. anguillarum*, concentration after 24 h in presence of *Phaeobacter* on GP and CC was, respectively, three and two logarithmic units lower than controls (Figure 6.3). Inhibition was maintained for five days. In presence of PB biofilters, inhibition started after 48 h, reaching inhibition levels of 30 – 60 % at the end of the experiment.

For *V. splendidus*, no inhibitory effect was observed when *Phaeobacter* was grown on PB (Figure 6.3). With porous supports, *V. splendidus* growth was inhibited in one or two logarithmic units, as compared to controls, reaching after 72 h inhibition levels of 20 and 35 % for GP and CC, respectively (Figure 6.3).

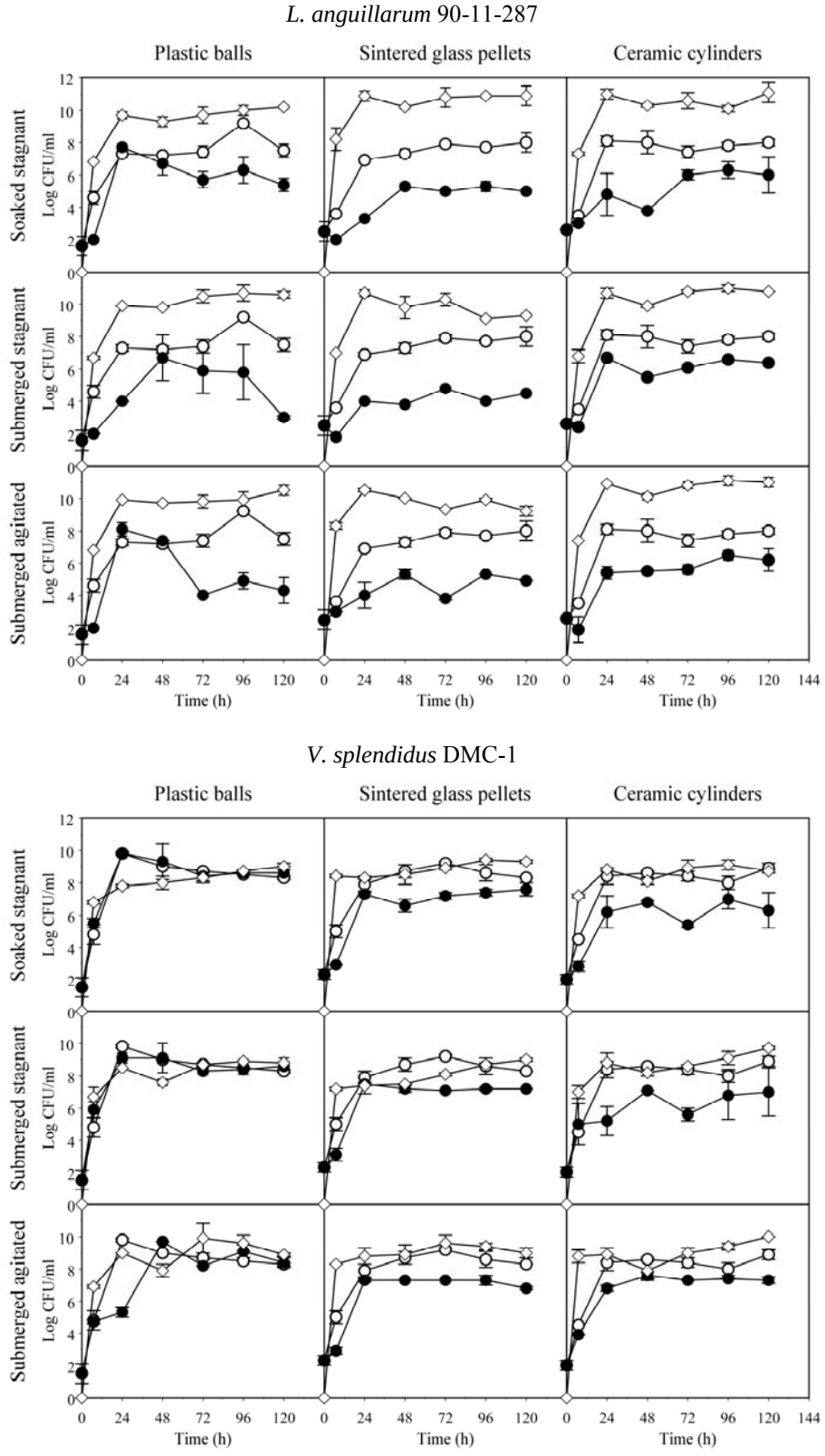

Figure 6.3. Inhibition of *Listonella anguillarum* 90-11-287 and *Vibrio splendidus* DMC-1 by *Phaeobacter* 27-4 immobilized on different supports. Pathogens concentration in presence (●) and absence (○) of *Phaeobacter*, as well as levels of *Phaeobacter* (◊) in the MB are shown. Data are mean and standard deviations of two independent assays.

In the biofilters, *Phaeobacter* grew on CC in one or two logarithmic units after 120 h (Table 6.3). In PB and GP *Phaeobacter* concentration diminished in one logarithmic unit or maintained the initial levels (Table 6.3). In all cases, *Phaeobacter* was released from the supports and grew in the MB, reaching a maximum of $10^9 – 10^{10}$ CFU·ml^{-1} between 7 and 24 h, which was maintained afterwards. Growth of *Phaeobacter* 27-4 in MB was lower in presence of *V. splendidus* (Figure 6.3).

At the end of the experiment (120 h), *L. anguillarum* and *V. splendidus* were detected attached to the three supports tested (Table 6.3). In controls, pathogens concentrations were higher on GP and CC ($10^6 – 10^7$ CFU cm^{-3}) than on PB ($10^4 – 10^5$ CFU cm^{-3}). The presence of *Phaeobacter* 27-4 reduced in one logarithmic unit the concentration of *L. anguillarum* attached to PB and CC, whereas the reduction was of almost five logarithmic units in GP. For *V. splendidus*, the concentration in controls and PB was similar and in GP and CC was one logarithmic unit lower.

6.3.3. Residence time of *Phaeobacter* 27-4 in biofilters maintained under turbot larval rearing conditions

Phaeobacter 27-4 was cultured in all supports under SbS conditions. The initial concentration of *Phaeobacter* 27-4 on PB and CC was similar (10^8 CFU·cm^{-3}) and one logarithmic unit higher on GP.

After the biofilters were submerged in green seawater, *Phaeobacter* 27-4 maintained for at least eleven days in the three supports tested but released faster from PB than from GP and CC (Figure 6.4). In PB, two different phases could be observed. In the first one, which would correspond to the detachment of the probiont from the biofilter (0 - 72 h), *Phaeobacter* concentration decreased constantly (slope (m) = -0.0376). In a second phase, the concentration of the probiotic in the support was maintained, decreasing slowly (m = -0.0027). A similar pattern was observed for GP, but with a lower slope in the first phase (m = -0.0202). For CC, those two phases could not be distinguished and a constant decrease (m = -0.0089) was observed.

The release of *Phaeobacter* from biofilters to the surrounding seawater occurred quickly in the first 6 h, reaching concentrations of about 10^5 bacteria·ml^{-1} (90 % of total bacteria) and decreasing differently afterwards depending on the support (Figure 6.4). The total numbers of probiotic bacteria introduced in the rearing tanks with the different

biofilters were $1.09 \cdot 10^{10}$ for PB, $8.14 \cdot 10^{11}$ for GP and $6.9 \cdot 10^{10}$ for CC. After 6 h, total numbers of *Phaeobacter* in the seawater was one logarithmic unit lower than those introduced at t_0 with the biofilters. Similarly to supports, two phases could be also observed in the seawater of PB and GP biofilters, with a decrease in the first 48 – 72 followed by a stabilization of the probiotic in the seawater. For CC, the decrease of *Phaeobacter* in seawater occurred constantly. The introduction of *Phaeobacter* biofilters did not affect total bacteria levels in seawater.

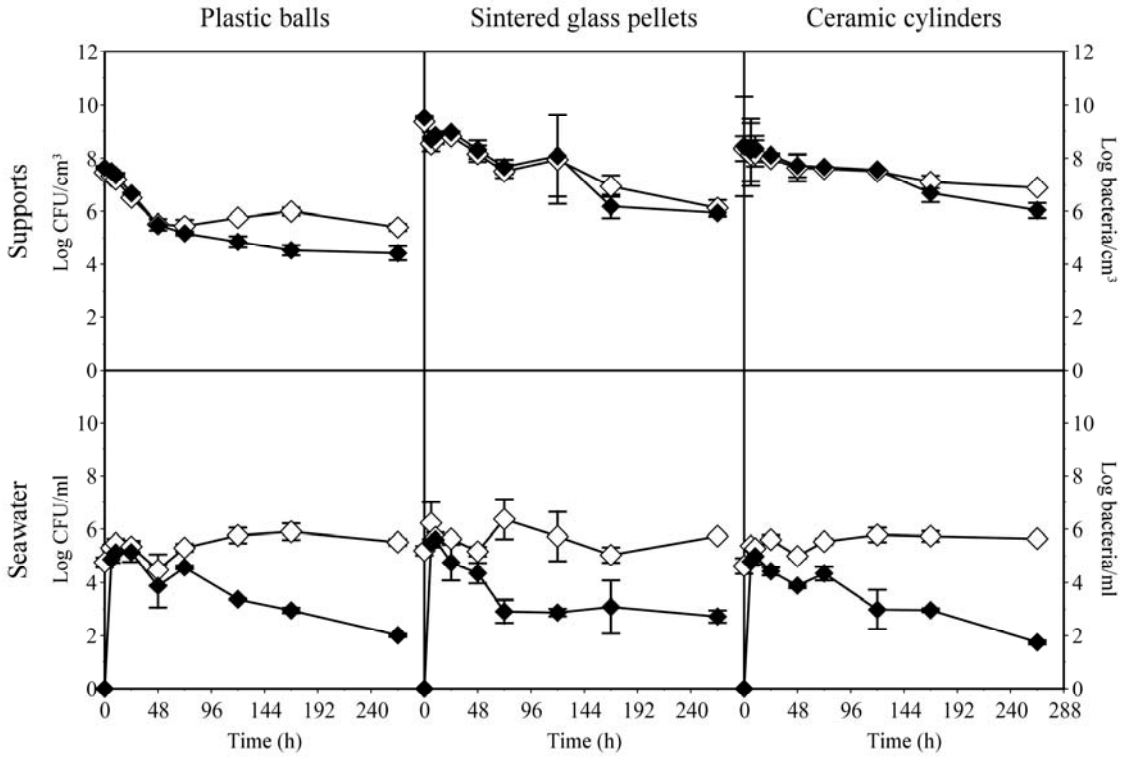

Figure 6.4. Residence time of *Phaeobacter* 27-4 in biofilters prepared with PB, GP and CC maintained under turbot rearing conditions and transference to the green seawater of tanks. Total bacteria counts from MA plates (◊) and *Phaeobacter* 27-4 levels from real-time PCR (♦) are represented. Data are mean and standard deviation of two independent assays and each assay value is the average of a technical duplicate.

Levels of *Phaeobacter* at the end of the trial (264 h) were one logarithmic unit lower on PB (10^5 bacteria·cm^{-3}) than on porous supports (10^6 bacteria·cm^{-3}). *Phaeobacter* accounted for more than 80 % of total bacteria in all supports for at least eleven days.

According to the longer permanence of the probiotic and to the better performance in the inhibition trials, porous supports seemed more adequate than the plastic one. CC, more resistant than GP, were selected for pilot-scale experiments.

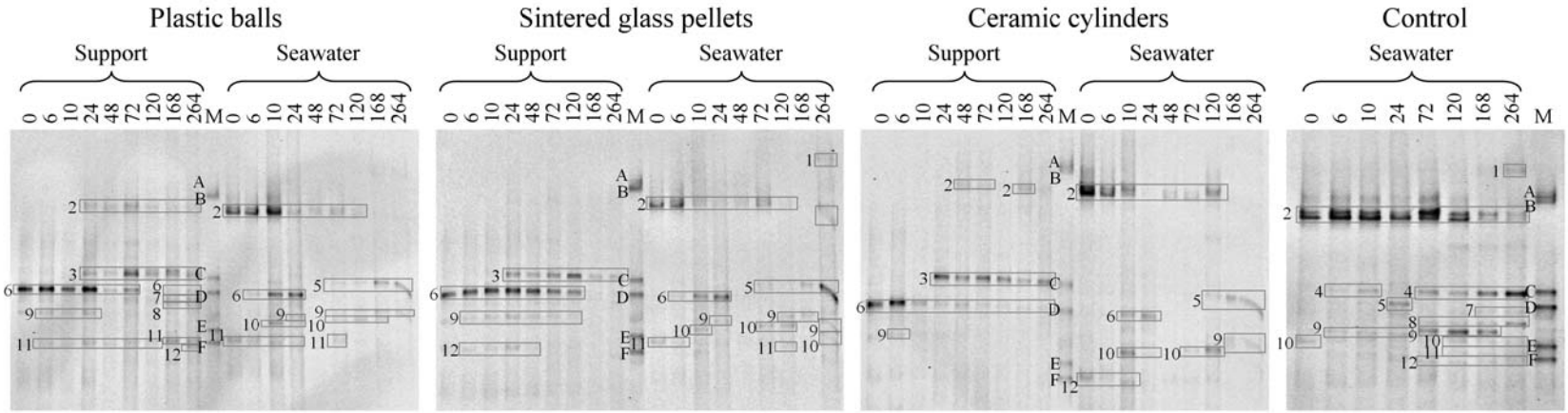

Figure 6.5. PCR-DGGE profiles of 16S rDNA fragments of biofilters and seawater samples throughout the maintenance experiment. M: marker (A: *Kordia algicida*, B: *Tenacibaculum discolor*, C: *Listonella anguillarum* 90-11-287, D: *Phaeobacter* 27-4, E: *Ruegeria mobilis*, F: *Flexibacter* sp)

6.3.3.1. Analysis of DGGE profiles

DGGE analysis was carried out to study the evolution of the biofilters and to monitor the changes produced by the introduction of the probiotic in the seawater of rearing tanks.

Biofilters prepared with PB showed a higher number of bands than those prepared with GP and CC, showing the last one the lowest number (Figure 6.5). A band with a migration pattern similar to *Phaeobacter* 27-4 (Band 6) was detected in biofilter and seawater samples taken from all tanks with biofilter but not in those from *Control* tanks. Band 2 may correspond with the microalgae *I. galbana* (Pintado *et al.*, in press). Some bands (Bands 4 and 9) were only present in seawater of *Control* tanks. Band 3 was detected in all biofilters from 24 h, showing increasing intensity with time but was absent in seawater samples.

Table 6.4. Range-weighted richness parameter of biofilters and seawater throughout maintenance experiment.

Sample	Time (h)	*Rr*			
		Control	PB	GP	CC
Biofilters	0	[a] -	0.05	0.05	0.05
	6	-	0.90	0.45	0.20
	10	-	0.90	0.45	0.05
	24	-	5.00	1.60	0.20
	48	-	3.20	1.60	0.60
	72	-	3.20	0.90	0.60
	120	-	1.80	0.90	0.20
	168	-	7.20	0.05	0.60
	264	-	9.80	0.05	0.20
Seawater	0	0.80	0.90	0.60	0.80
	6	1.35	1.80	1.35	0.80
	10	1.35	3.20	0.90	3.20
	24	1.35	2.40	1.35	0.40
	48	-	0.05	0.05	0.05
	72	5.00	5.00	1.35	0.60
	120	9.80	2.40	3.75	1.35
	168	12.80	0.45	0.40	0.20
	264	12.80	0.20	5.00	0.20

[a] -: Not analysed

Rr was always < 10 in both biofilters and seawater with exception of seawater of *Control* tanks at the end of the experiment.and showed the lowest values in tanks with CC biofilters (Table 6.4). Changes on bacterial microbiota between samples were established using a moving-window analysis in biofilters and seawater (Figure 6.6). In PB biofilters, the most important changes occurred at 48 h (51 %). CC biofilters showed a first shift of 92 % at 24 h and a second one at 168 h (33 %). In GP biofilters, less pronounced changes were observed.

Seawater of *Control* tanks showed increasing differences with time. Differently, in presence of biofilters major changes were only detected at 24 – 48 h (72 – 97 %) and at 120 h (43 – 45 %).

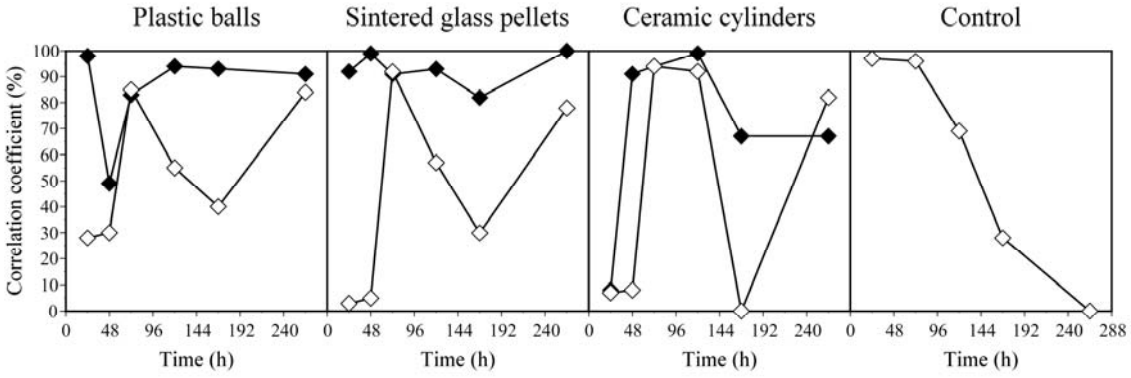

Figure 6.6. Moving-window analysis based on DGGE profiles of biofilters (♦) and seawater (◊) of maintenance experiment. Each data point in the graph is a comparison of two dates.

6.3.4. Effect of matured *Phaeobacter* 27-4 biofilters against *Listonella anguillarum* 90-11-287 maintained under turbot larval rearing conditions

The capability of *Phaeobacter* 27-4 biofilters to antagonize *L. anguillarum* 90-11-287 was checked in normal non axenic turbot larval rearing conditions, in absence or presence of high nutrients (MB) level. *Phaeobacter* 27-4 cultures were prepared in CC-SbS conditions, which ensured the formation of a well developed biofilm (Figure 6.1), the antagonism of *L. anguillarum* and *V. splendidus* (Figures 6.2 and 6.3) and a longer permanence of the probiont in the tanks when maintained under larval rearing conditions (Figure 6.7).

The introduction of *Phaeobacter* biofilters inhibited *L. anguillarum* both in presence and absence of MB. Inhibition started at 6 h in tanks without MB addition and at 24 h in the tanks with MB. In treatment *La*, levels of *L. anguillarum* were stable (10^4 – 10^5 CFU·ml^{-1}) for three days and decreased one or three logarithmic units afterwards, depending on the addition or not of MB (Figure 6.7).

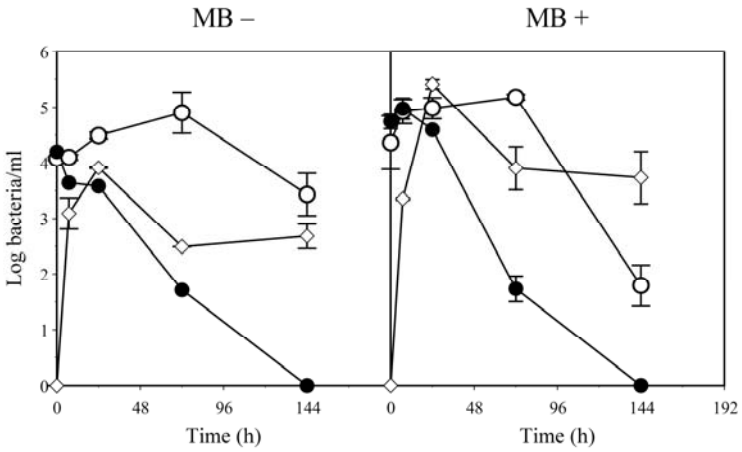

Figure 6.7. Effect of *Phaeobacter* 27-4 biofilters on *Listonella anguillarum* 90-11-287 in green seawater tanks maintained under turbot rearing conditions. Levels of *L. anguillarum* in presence (●) and absence (○) of *Phaeobacter* biofilters, as well as, of *Phaeobacter* bacteria transferred from the biofilters to the seawater of tanks (◊) are shown. Data are mean and standard deviation of two independent assays and each assay value is the average of a technical duplicate.

In treatment *LaBPh* the level of *L. anguillarum* was progressively reduced and undetectable by real-time PCR at 144 h (Figure 6.7). According to the slope of disappearance of *L. anguillarum* the calculated level would be 0 at 96 h. By then, the concentration of *L. anguillarum* in seawater of the tanks without biofilter was about 10^4 bacteria·ml^{-1}. *L. anguillarum* was not detected in biofilters throughout the experiment, in any of the treatments.

Phaeobacter concentration on the matured biofilters introduced in *BPh* and *LaBPh* tanks (0 h) was $(2.11 \pm 0.12)\cdot10^5$ CFU·cm^{-3}. The release of *Phaeobacter* from biofilters to seawater occurred during the first 24 h, reaching a maximum concentration of 10^5 and 10^3 CFU·ml^{-1} in presence and absence of MB, respectively (Figure 6.7). After 72 h, the concentration of *Phaeobacter* 27-4 in seawater remained at levels of 10^2 bacteria·ml^{-1} in absence of MB and 10^3 bacteria·ml^{-1} in presence of MB. In the biofilters, concentration at the end of the experiment was $(1.84 \pm 0.31)\cdot10^6$ bacteria·cm^{-3} without MB addition and $(5.73 \pm 0.14)\cdot10^6$ CFU·cm^{-3} in presence of MB.

Total bacteria concentrations in seawater, obtained from MA plate counts, were similar in all treatments, ranging from $(1.22 \pm 0.20)\cdot10^4$ CFU·ml^{-1} at 0 h to $(1.24 \pm 0.26)\cdot10^5$ CFU·ml^{-1} at 144 h. Total bacteria in biofilters at the end of the trial was $(8.35\pm 0.36)\cdot10^6$ and $(3.20 \pm 0.14)\cdot10^7$ CFU·cm^{-3} in absence and presence of MB, respectively.

Total *Vibrionaceae* reached the highest proportion with respect total bacteria (> 50 %) in treatment *La* at the beginning of the experiment, both in presence and absence of MB. In *BPh* treatment, *Vibrionaceae* were only detected at 24 h in presence of MB (2 % of total bacteria). *La* and *LaBPh* treatments showed similar concentrations of *Vibrionaceae* (10^4 – 10^5 CFU·ml^{-1}) at the beginning of the experiment in seawater independently of MB addition. The presence of the biofilter (*LaBPh*), promoted the disappearance of the *Vibrionaceae* in seawater after 24 h, whereas in *La* treatment *Vibrionaceae* remained detectable until 72 h. In samples taken from the biofilters of *BPh* and *LaBPh* treatments at the end of the trial (144 h) the *Vibrionaceae* concentration was $(2.49 \pm 0.43)\cdot10^2$ CFU·cm^{-3} in absence of MB and 99.8 ± 0.35 CFU·cm^{-3} in MB treatment. In treatments *Control* and *La*, *Vibrionaceae* attained $(1.85 \pm 0.08)\cdot10^4$ CFU·cm^{-3} in tanks with MB or $(4.34 \pm 0.26)\cdot10^3$ CFU·cm^{-3} in tanks without MB.

6.3.5. *In vivo* probiotic effect of *Phaeobacter* 27-4 biofilters on turbot larvae

To assess the effectiveness of *Phaeobacter* 27-4 biofilters in the protection of turbot larvae against *Vibrionaceae* infections, the larvae were challenged with *L. anguillarum* 90-11-287, introduced via rotifers, in absence or presence of biofilters in the rearing tanks. Biofilters significantly reduced the mortalities caused by *L. anguillarum* ($p < 0.05$) (Figure 6.8A). Accumulated mortality in ten days old larvae infected with *L. anguillarum* was 76 ± 20 %, whereas the mortality in larvae infected with *L. anguillarum* and treated with *Phaeobacter* was similar to that of controls (35 – 40 %).

Initial concentration of *Phaeobacter* 27-4 in matured biofilters was $(4.26 \pm 1.10)\cdot10^6$ bacteria·cm^{-3}, which was maintained until the end of the trial. The concentration of *L. anguillarum* in rotifers used to infect turbot larvae at days four, six and eight post-hatching, was $(1.85 \pm 0.06)\cdot10^2$, $(8.20 \pm 0.09)\cdot10^2$ and $(5.61 \pm 0.00)\cdot10^3$ bacteria·rotifer^{-1}, respectively. *Phaeobacter* and *L. anguillarum* were not detected by real-time PCR in uninfected rotifers used to feed turbot larvae at days three, five, seven and nine.

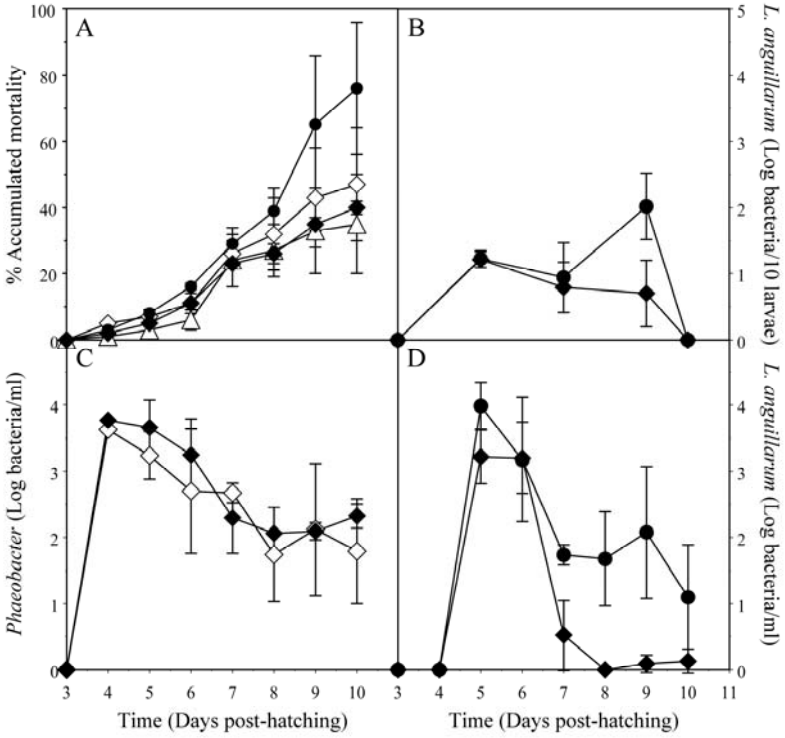

Figure 6.8. Probiotic effect of *Phaeobacter* 27-4 biofilters on turbot larvae challenged with *Listonella anguillarum* 90-11-287. (A) Percentage of accumulated mortalities (Δ: *Control*, ◊: *BPh* treatment, ●: *La* treatment, ♦: *LaBPh* treatment). (B) Levels of *L. anguillarum* in infected larvae treated (♦) or not (●) with *Phaeobacter* 27-4. (C) Levels of *Phaeobacter* 27-4 in seawater of the tanks with turbot larvae challenged (♦) or not (◊) with *L. anguillarum*. (D) Levels of *L. anguillarum* in seawater of *La* (●) and *LaBPh* (♦) tanks. Data are mean and standard deviation of two independent assays and each assay value is the average of a technical duplicate.

In larvae, *Phaeobacter* was not detected in treatments *BPh* and *LaBPh* whereas *L. anguillarum* was detected in all larvae treated with the pathogen (Figure 6.8B). Concentrations of *L. anguillarum* in five days old larvae were almost the same in *La* and *LaBPh* treatments (40 bacteria·10 larvae^{-1}). *L. anguillarum* concentration in larvae treated with *Phaeobacter* decreased, whereas, the level increased to 10^2 bacteria·10 larvae^{-1} at day nine in *La* treatment. In both cases, *L. anguillarum* was undetectable in larvae at the end of the experiment (day ten).

In seawater, *Phaeobacter* was found in treatments *BPh* and *LaBPh* (10^3 bacteria·ml^{-1}), 24 h after biofilters were transferred to the experimental tanks (day four post-hatching) (Figure 6.8C). Concentration of *Phaeobacter* in seawater decreased until day eight, and then remained at 10^2 bacteria·ml^{-1} until the end of the trial (day ten post-hatching).

Table 6.5. Total bacteria and *Vibrionaceae* plate counts during the challenge trial of *Phaeobacter* 27-4 biofilters on turbot larvae infected by *Listonella anguillarum* 90-11-287 via rotifers

		Larvae (Log CFU·10 larvae^{-1})				Seawater (Log CFU·ml^{-1})			
	Days post hatching	*Control*	*BPh*	*La*	*LaBPh*	*Control*	*BPh*	*La*	*LaBPh*
Total bacteria	3	2.4 ± 0.43	2.00 ± 0.00	2.38 ± 0.22	2.50 ± 0.51	5.49 ± 0.08	5.44 ± 0.43	5.54 ± 0.00	5.23 ± 0.04
	5	[a] NA	NA	NA	NA	6.41 ± 0.04	6.52 ± 0.58	7.04 ± 0.05	6.40 ± 0.02
	7	3.98 ± 0.24	4.81 ± 0.13	4.76 ± 0.32	5.33 ± 0.45	6.43 ± 0.07	6.91 ± 0.08	6.77 ± 0.01	6.23 ± 0.22
	9	NA	NA	NA	NA	5.61 ± 0.23	5.35 ± 0.04	5.99 ± 0.04	5.42 ± 0.13
	10	5.66 ± 0.65	4.92 ± 0.13	4.64 ± 0.10	4.65 ± 0.38	5.59 ± 0.37	6.17 ± 0.51	6.14 ± 0.10	5.42 ± 0.13
Total *Vibrionaceae*	3	1.18 ± 0.21	1.45 ± 0.40	1.34 ± 0.38	1.53 ± 0.50	4.18 ± 0.21	4.93 ± 0.62	3.70 ± 0.00	4.56 ± 0.00
	5	NA	NA	NA	NA	5.65 ± 0.81	5.47 ± 1.47	6.41 ± 0.16	5.34 ± 0.57
	7	3.65 ± 0.07	4.42 ± 0.11	4.55 ± 0.56	4.31 ± 0.08	4.48 ± 0.00	5.98 ± 1.40	5.30 ± 0.00	5.02 ± 0.15
	9	NA	NA	NA	NA	4.00 ± 0.00	ND	5.06 ± 0.08	ND
	10	4.57 ± 0.97	4.76 ± 0.39	4.28 ± 0.03	4.40 ± 0.43	ND	ND	5.23 ± 0.27	ND

[a] NA: Not analysed. *Control*: larvae were fed on rotifers enriched with microalgae; *BPh*: larvae were fed on rotifers enriched with microalgae and a biofilter was placed in the tanks; *La*: larvae were fed on rotifers loaded with *L. anguillarum* (days four. six and eight); *LaBPh*: larvae were fed on rotifers loaded with *L. anguillarum* (days four. six and eight) and a biofilter was placed in the tanks.

In samples from rotifers taken at day ten, *Phaeobacter* and *L. anguillarum* were not detected in any of the tanks.

The concentration of *L. anguillarum* in seawater of *La* and *LaBPh* treatments increased to a maximum value ($10^3 – 10^4$ bacteria·ml^{-1}) two days after first feeding (day four post-hatching) (Figure 6.8D) and decreased afterwards, stabilizing in > 10^4 bacteria·ml^{-1} from day seven for *La* treatment and becoming undetectable or in a very low concentration, from day eight in treatment *LaBPh* (Figure 6.8C).

Similar values for total bacteria (MA) and total *Vibrionaceae* (TCBS) concentration were detected in larvae by plate counting (Table 6.5). *Vibrionacea*e covered more than 40 % of total bacteria at the end of the trial in all treatments.

In seawater, total bacteria concentration was similar ($10^5 – 10^6$ CFU·ml^{-1}) in all treatments at any time (Table 6.5). *La* treatment had the highest concentration in total *Vibrionaceae* ($10^5 – 10^6$ CFU·ml^{-1}) whereas *Control*, *BPh* and *LaBPh* treatments had 10^5 and 10^4 CFU·ml^{-1} of total *Vibrionaceae* at days seven and nine, respectively. *Vibrionaceae* were not detected in *BPh* and *LaBPh* tanks from day nine and from day ten in *Control* tanks.

In the biofilters, the concentration of total bacteria at the end of the trial (day ten) was of around 10^7 CFU·cm^{-3} in all treatments. Total *Vibrionaceae* in *Phaeobacter* biofilters was one logarithmic unit lower ((2.84 ± 1.77)·10^3 CFU·cm^{-3}) than in *Control* and *La* treatments ((1.95 ± 2.56)·10^4 CFU·cm^{-3}). *L. anguillarum* was not detected in biofilters at the end of the trial in any of the cases.

The presence of the *Phaeobacter* biofilters reduced turbidity (OD_{600}), from 0.055 ± 0.024 and 0.075 ± 0.024 in treatments *Control* and *La*, respectively, to 0.033 ± 0.013 in *BPh* and 0.023 ± 0.004 in *LaBPh*.

6.3.5.1. Analysis of DGGE profiles

In three days old larvae, four predominant bands were detected (Bands 3, 4, 9 and 11). The number of bands increased to eight (*LaBPh*) or eleven (*Control*, *BPh* and *La*) during the experimental period. No bands with migration corresponding to *L. anguillarum* 90-11-287 and *Phaeobacter* 27-4 were detected in larvae (Figure 6.9).

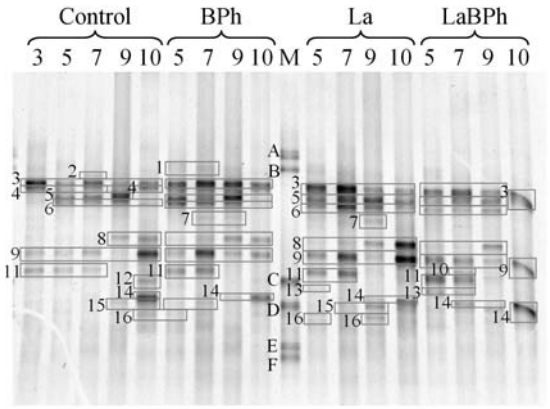

Figure 6.9. PCR-DGGE profile of 16S rDNA fragments of turbot larvae from treatments *Control*, *BPh*, *La* and *LaBPh*. M: marker (A: *Kordia algicida*, B: *Tenacibaculum discolor*, C: *Listonella anguillarum* 90-11-287, D: *Phaeobacter* 27-4, E: *Ruegeria mobilis*, F: *Flexibacter* sp).

A few predominant bands (3, 5, 6 and 9) appeared in larvae from all treatments and others (1, 2, 4, 10) were detected only in some of the treatments. No clear differences were observed between treatments. Larvae profiles clustered together in function of time and grouped with the profiles of rotifers enriched with microalgae and used to feed the larvae (Figure 6.10).

In rotifers, profiles from non infected *I. galbana*-enriched rotifers (*RotIg*) were similar (Figure 6.11) and clustered together (Figure 6.10). Some differences were observed in the profiles obtained from rotifers loaded with *L. anguillarum* (*RotLa*) used for the infection of the larvae. This indicates variability in the bioencapsulation of the pathogen and is in accordance with the results obtained from the quantification of *L. anguillarum* by real-time PCR. *RotLa* profiles showed a single predominant band (band 9) with a migration pattern similar to *L. anguillarum* (Band C of the marker) at days six and eight, resulting in the separation of those profiles from the other rotifers in the dendrogram. At day four, the infected rotifers showed a less predominant band corresponding to *L. anguillarum*, being the profile more similar to non-infected rotifers (*RotIg*) and clustered together. Rotifers obtained from the tanks at day ten post-hatching showed a band (Band 7), already present in the rotifers *RotIg* before being added to the tanks, which was predominant. Bands 2, 3 and 10 were also detected in rotifers *RotIg*. Those profiles clustered with samples taken from seawater at the same time (Figure 6.10), indicating that some of the new bands present in rotifers may come from the seawater of rearing tanks.

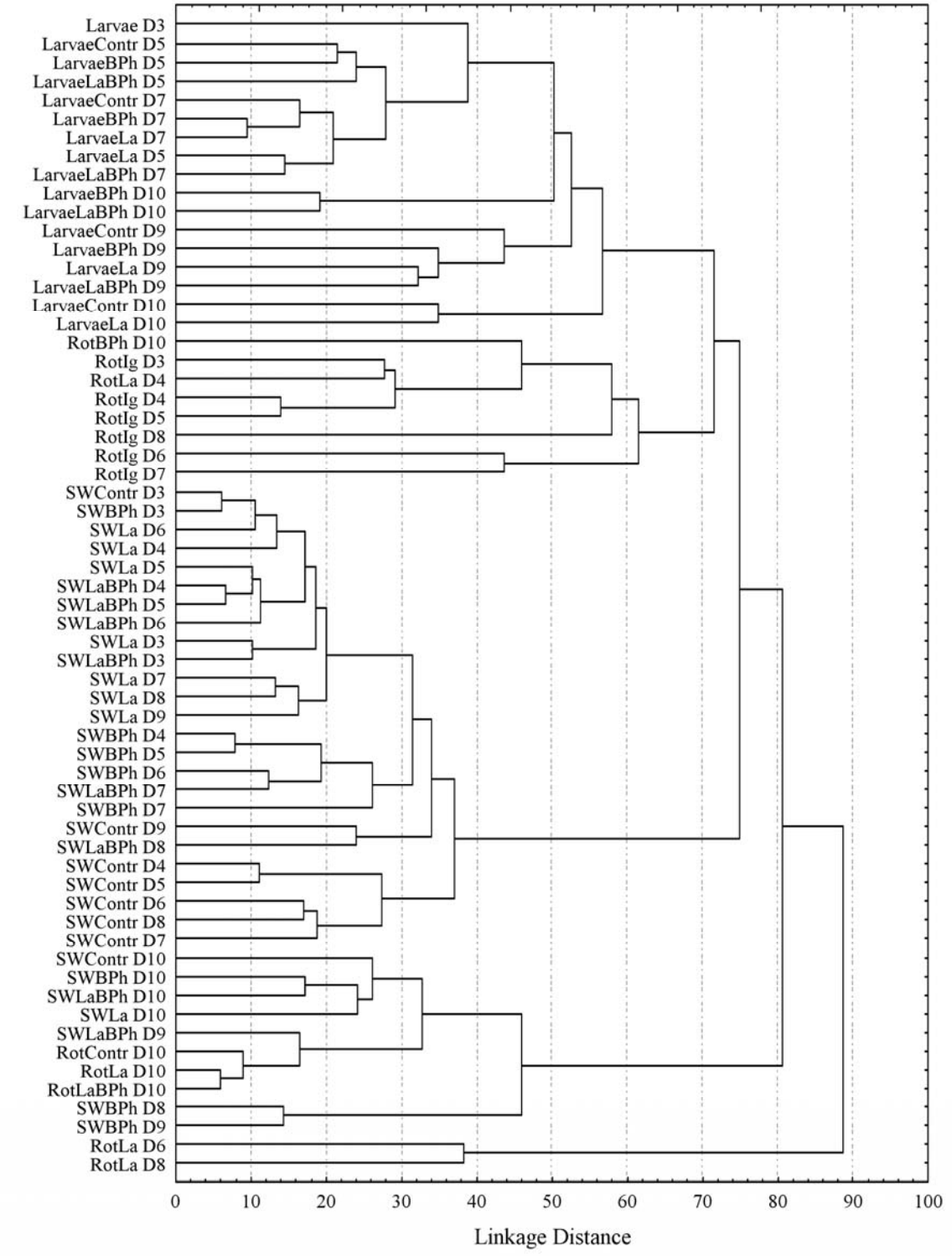

Figure 6.10. Euclidean-distance dendrogram generated from the DGGE profiles of turbot larvae and seawater samples taken from the treatments *Control*, *BPh*, *La* and *LaBPh*, of rotifers taken from the rearing tanks at day ten post-hatching and rotifers enriched with *I. galbana* and rotifers loaded with *L. anguillarum*. The dendrogram was determined by the unweighted-pair group method using average linkages.

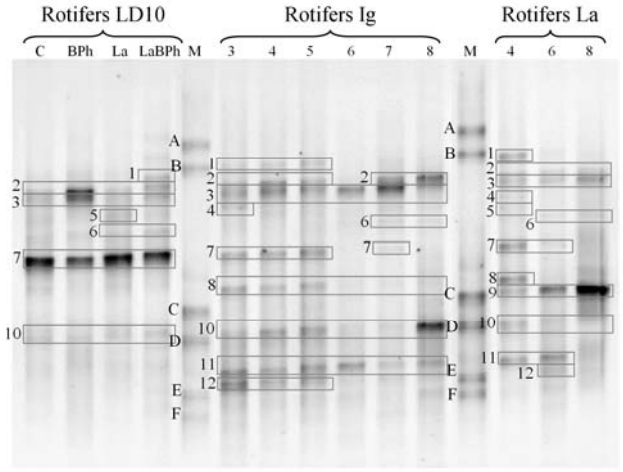

Figure 6.11. PCR-DGGE profile of 16S rDNA fragments of rotifers samples from tanks at day 10 post-hatching (Rotifers LD10), of rotifers enriched with *I. galbana* (Rotifers Ig) and of rotifers loaded with *L. anguillarum* (Rotifers La). M: marker (A: *Kordia algicida*, B: *Tenacibaculum discolor*, C: *Listonella anguillarum* 90-11-287, D: *Phaeobacter* 27-4, E: *Ruegeria mobilis*, F: *Flexibacter* sp).

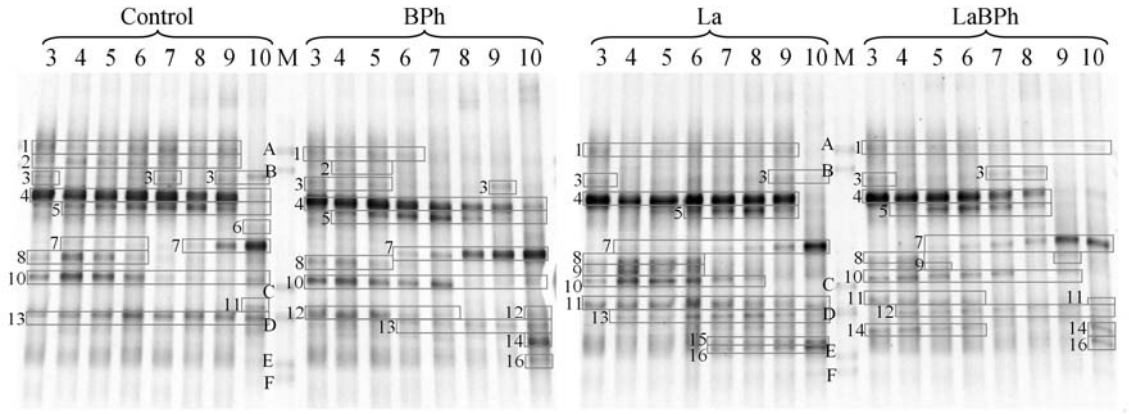

Figure 6.12. PCR-DGGE profile of 16S rDNA fragments of seawater samples taken from treatments *Control*, *BPh*, *La* and *LaBPh*. M: marker (A: *Kordia algicida*, B: *Tenacibaculum discolor*, C: *Listonella anguillarum* 90-11-287, D: *Phaeobacter* 27-4, E: *Ruegeria mobilis*, F: *Flexibacter* sp).

DGGE profiles of samples from seawater (Figure 6.12) showed a similar number of bands in all treatments. In treatments *BPh* and *LaBPh*, a band with a similar migration pattern as *Phaeobacter* 27-4 was detected from day 4 (Band 12). Band 4 was detected throughout the trial and may correspond to the *I. galbana* plastid DNA (Pintado *et al.*, in press). Bands 1, 3, 8 and 10 were predominant at the beginning of the experiment. Bands 5 or 7 became predominant at intermediate or longer times, respectively. Band 9 was only detected in *La* and *LaBPh* tanks and bands 12 and 14 only in treatments *BPh* and *LaBPh*.

Samples from seawater clustered independently from larvae and non infected rotifers (Figure 6.10). Within the seawater profiles grouping, three main clusters could be distinguished: tanks with *L. anguillarum* (*La* and *LaBPh*), tanks with the biofilter and without the pathogen (*BPh*), and *Control* tanks. A separated group clustered samples from seawater and rotifers taken from the rearing tanks at the end of the experiment (day ten post-hatching).

Range-weighted richness (*Rr*), dynamics (*Dy*) and functional organization (*Fo*) were calculated to analyse the influence of *L. anguillarum* 90-11-287 and *Phaeobacter* 27-4 in larvae bacterial community.

Rr was always <10 in larvae, independently of the treatment (Table 6.6), indicating that bacterial diversity in turbot larvae was low. In seawater, *Rr* values were higher than in larvae and some differences were observed, depending on the treatment. Seawater of *Control* tanks showed values of 9.80 – 12.80 throughout the trial.

In *La* treatment, an increase with respect to *Control* tanks was observed with *Rr* values of 12.80 – 16.20 from the beginning until day ten when the lowest value of this treatment was achieved (7.35). The presence of *Phaeobacter* 27-4 biofilters (Treatments *BPh* and *LaBPh*) resulted in a decrease of seawater microbial diversity (3.75 – 9.80) from days six or seven. In *BPh* treatment, medium *Rr* values were recorded at the end of the experiment (day ten).

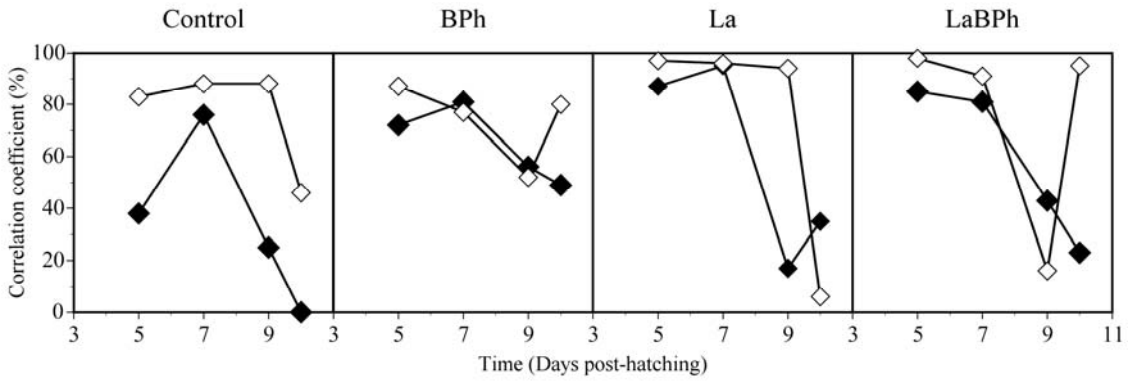

Figure 6.13. Moving-window analysis based on DGGE profiles of turbot larvae (♦) and seawater (◊) of treatments *Control*, *BPh*, *La* and *LaBPh*. Each data point in the graph is in itself a two days comparison, as it represents the correlation between the samples of day x and day x–2.

Table 6.6. Range-weighted richness parameter of turbot larvae and seawater throughout *in vivo* challenge trial.

Treatment	Days post-hatching	Larvae		Seawater	
		Rr	[a] Rank	*Rr*	Rank
Control	3	1.60	L	9.80	L
	4	NA	NA	12.80	M
	5	3.60	L	12.80	M
	6	NA	NA	12.80	M
	7	4.90	L	9.80	L
	8	NA	NA	9.80	L
	9	3.60	L	12.80	M
	10	8.10	L	12.80	M
BPh	3	1.60	L	7.20	L
	4	NA	NA	12.80	M
	5	8.10	L	12.80	M
	6	NA	NA	9.80	L
	7	8.10	L	7.20	L
	8	NA	NA	5.00	L
	9	4.90	L	7.20	L
	10	3.60	L	12.80	M
La	3	1.60	L	12.80	M
	4	NA	NA	16.20	M
	5	6.40	L	16.20	M
	6	NA	NA	16.20	M
	7	4.90	L	16.20	M
	8	NA	NA	16.20	M
	9	8.10	L	16.20	M
	10	3.60	L	7.35	L
LaBPh	3	1.60	L	9.80	L
	4	NA	NA	18.80	M
	5	4.90	L	16.20	M
	6	NA	NA	12.80	M
	7	8.10	L	9.80	L
	8	NA	NA	9.80	L
	9	6.40	L	3.75	L
	10	0.90	L	5.40	L

[a] L: Low microbial diversity (*Rr* < 10); M: Intermediate microbial diversity (30 > *Rr* >10); NA: Not analysed.

Changes on bacterial microbiota between samples (every two days) were established using a moving-window analysis in larvae and seawater (Figure 6.13). In all cases, the most important changes occurred from day seven. Larvae of *Control* tanks showed a shift of 62 % in the first 48 h (day five) and a second shift about 75 and 100 % at days nine and ten. Less pronounced changes were observed in larvae of treatment *BPh* where changes were 28 % at day five and 51 % at day ten. In treatments with *L. anguillarum* (*La* and *LaBPh*) similar shifts were observed, being about 57 – 83 % at day nine and 65 – 77 % at day ten.

Major changes in seawater occurred at days nine and ten in treatments *Control* and *La* and from day seven in treatments with *Phaeobacter* 27-4 biofilter (*BPh* and *LaBPh*). As for larvae, community shifts were less pronounced in treatment *BPh* as demonstrated by the shorter fluctuations in the moving-window analysis plot.

Average values for these rates of change were calculated and expressed as rates of change [$\Delta_{t\ (2\ days)}$]. The total community changed more rapidly in larvae of treatment *Control* (53.67 ± 26.50 %) and in seawater of treatment *LaBPh* (31.67 ± 45.46 %) than in larvae (30 – 33 %) and seawater (4 – 28 %) from the other treatments, being the lowest value observed in treatment *L*a (4.33 ± 1.53 %).

In order to graphically represent the structure of the bacterial community for larvae and seawater, Pareto-Lorenz evenness curves were constructed based on DGGE profiles (Figure 6.14). In all treatments, 20 % of the bands present in DGGE profiles of larvae corresponded with 43 – 53 % (on average 49 %) of the cumulative band intensities and with 59 – 69 % (on average 64 %) in seawater.

6.4. Discussion

In a previous work, Planas *et al.* (2006) demonstrated the *in vivo* probiotic effect of *Phaeobacter* 27-4 on turbot larvae, when delivered to larvae bioencapsulated in rotifers. Nevertheless, this probiotic strain did not colonize permanently turbot larvae or rotifers (Pérez-Lorenzo, 2006; Pintado *et al.*, in press; Planas *et al.*, 2006), meaning that repeated additions would be needed to maintain effective levels of the probiont in the rearing system.

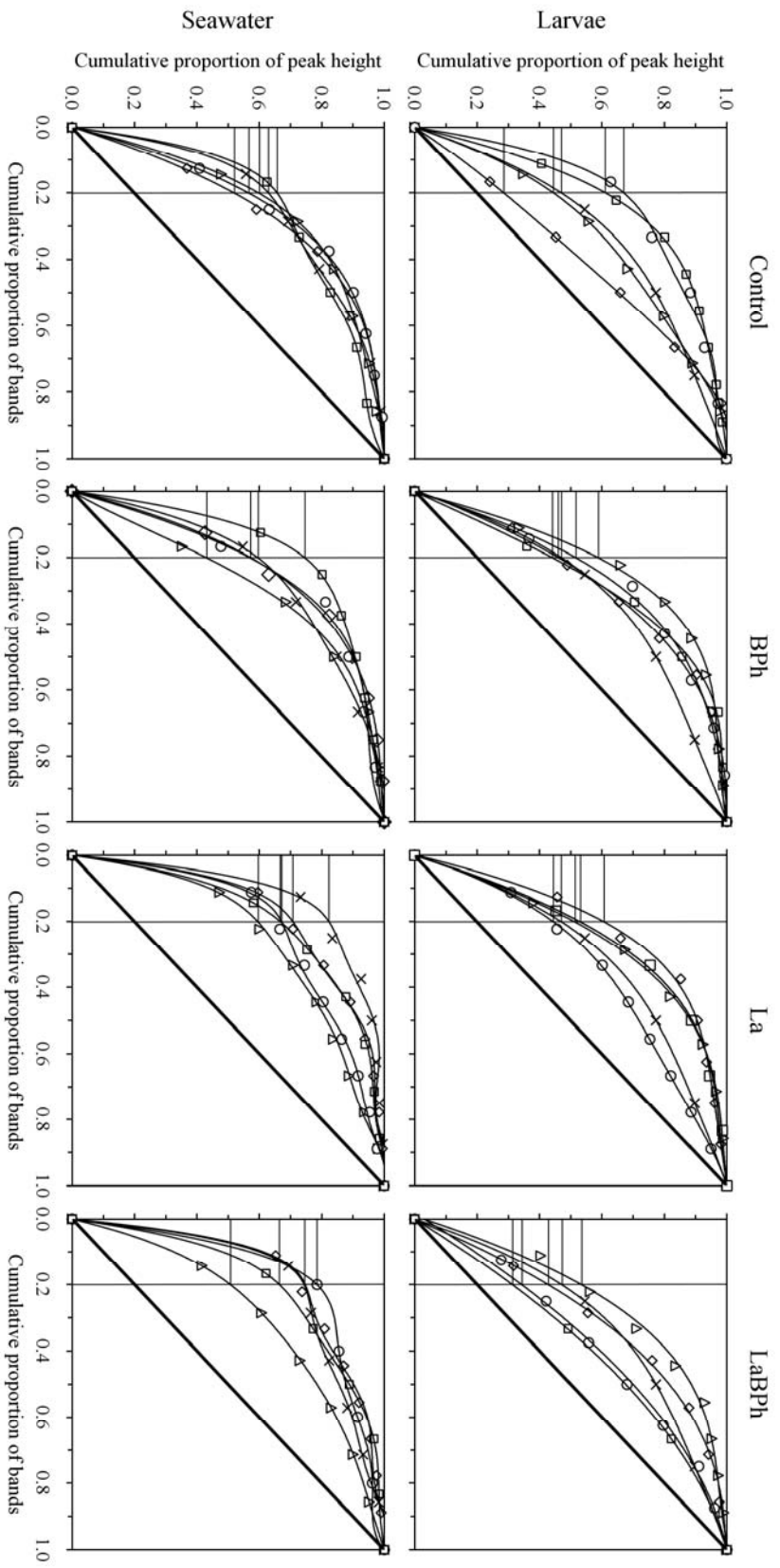

Figure 6.14. Pareto-Lorenz distribution curves based on PCR-DGGE analysis on day three (×), five (◊), seven (Δ), nine (○) and ten (□) after larval hatching of larvae and seawater of treatments *Control*, *BPh*, *La* and *LaBPh*. The dashed vertical line at the 0.2x axis level is plotted to evaluate the range of the Pareto values.

Based on the capability of *Phaeobacter* 27-4 to attach to surfaces and to form biofilms (Bruhn *et al.*, 2006), the present work studied the immobilization of *Phaeobacter* 27-4 on biofilters as an alternative strategy, which would guarantee the permanence of the probiotic bacteria in the rearing systems without continuous additions of the probiont.

Biofilters have been routinely used to maintain nitrifying bacteria in rearing systems and to improve seawater quality (Crab *et al.*, 2007; Michaud *et al.*, 2006; Salvesen *et al.*, 1999; Skjermo *et al.*, 1997). Verschuere *et al.* (2000) broadened the definition of probiotics in aquaculture to include bacteria which can exert their beneficial effect on the host by modification of the microbial community associated not only to fish but also to the environment (seawater).

In the present work, it has been demonstrated the *in vivo* probiotic effect of biofilters with *Phaeobacter* 27-4, increasing the survival on turbot larvae challenged with *L. anguillarum* 90-11-287.

6.4.1. Culture of *Phaeobacter* 27-4 on different supports and conditions

In most cases, microorganisms attach more rapidly to hydrophobic no-polar surfaces (e.g. plastics) than to hydrophilic materials, such glass or metals (Pringle and Fletcher, 1983; Bendinger *et al.*, 1993). In the present study *Phaeobacter* 27-4 was able to grow in the three materials tested. Influence of culture conditions was higher on PB than on porous supports, with a weaker attachment to plastic than to sintered glass and ceramic supports, reflected by the pronounced decrease of the probiotic concentration on PB-SbA after 96 h.

Porous supports showed the highest *Phaeobacter* 27-4 concentration when cultured in SkS and SbA conditions, situations in which aeration was favoured. These results agree with those of Bruhn *et al.* (2005), who reported that *Phaeobacter* 27-4 cultured with agitation reaches higher concentrations than in stagnant conditions

Bacteria attachment was influenced by culture conditions in all supports and did not correlate with bacterial concentration in supports, indicating that bacteria could be growing in the liquid media inside the interstices of supports.

Few rosette-shaped microcolonies were observed in PB-SbS, PB-SkS and in GP-SbS. A well developed multilayer biofilm with rosette-shaped microcolonies covering the outer surface of supports was only observed on CC-SbS. Characklis *et al.* (1990) pointed out that microbial colonization appears to be enhanced by surface roughness due to a lower number of shear forces and a greater area. A higher surface: volume ratio (Table 6.1) and a higher roughness observed by SEM (Figure 6.1) can be favouring biofilm formation on ceramic support. These results are in agreement with those of Bruhn *et al.* (2007) who reported that stagnant conditions promoted rosettes formation and the development of a biofilm.

Pigment formation by *Phaeobacter* 27-4 in the present study was low in CC cultures, even under the culture conditions favouring the formation of a biofilm. Furthermore, pigment present in filtered *Phaeobacter* culture supernatants was not significantly adsorbed by sintered glass and ceramic supports. These results are in disagreement with those previously reported by Bruhn *et al.*, (2005), who found a relation between pigment production by *Phaeobacter* 27-4 and the biofilm formation in the air-liquid interface.

6.4.2. *In vitro* inactivation and inhibition of *Listonella anguillarum* 90-11-287 and *Vibrio splendidus* DMC-1 by *Phaeobacter* 27-4 biofilters

L. anguillarum is more sensitive to the antagonistic compound produced by *Phaeobacter* 27-4 than *V. splendidus* (Bruhn *et al.*, 2005). However, in the present study, the inactivation caused by the probiotic biofilters in absence of nutrients reached, in most cases, similar levels for both pathogens and only few differences were detected due to support or culture conditions.

Differently, in experiments reproducing the high nutrients concentration in rearing tanks, the inhibition of *L. anguillarum* was higher than for *V. splendidus*, which grew more than *L. anguillarum* and induced a lower growth of *Phaeobacter* 27-4 in MB. These results suggest an important role of the competition for nutrients and agree with those reported by Vázquez *et al.* (2004), who found that the maximum specific growth rate is higher for *V. splendidus* than for *L. anguillarum* and *Phaeobacter* 27-4.

Both pathogens were able to colonize all supports. Similarly, Bourne *et al.* (2006) have found that *Vibrionaceae* are able to colonize fibreglass slides introduced in a

tropical rock lobster larval rearing tank, becoming the dominant species in the formed biofilm. In the present study, the presence of *Phaeobacter* in CC and GP biofilters seems to diminish their colonization by pathogens. Similarly, a *Phaeobacter gallaeciensis* strain was able to colonize and out-compete a pre-established biofilm on algae (Rao *et al.*, 2006).

As previously suggested by other authors, in the study of probiotic mechanisms (Tihn *et al.*, 2008), control experiments with a mutant *Phaeobacter* 27-4 strain lacking the capability to produce the antagonistic compound would help to elucidate if the mechanism of action is based mainly in antagonism or in competence.

Another aspect to be considered is whether the antagonistic effect is exerted predominantly by the probiotic in seawater/culture medium or by the probiotic attached to the biofilters. The level of *Phaeobacter* detected in seawater (released from the biofilter) in the inactivation trial was similar in all cases, suggesting that the effect may be also exerted by the probiotic in seawater. This would explain the small differences observed between the different supports and culture conditions. In presence of nutrients, although similar concentrations of the probiotic in the culture medium were also observed for the different treatments, an effect depending on the supports was observed on pathogens inhibition. These results indicate that the presence of the biofilter would have more influence on the inhibition of growth than in the inactivation of the pathogens.

6.4.3. Permanence of *Phaeobacter* 27-4 in biofilters and effect of matured biofilters against *Listonella anguillarum* 90-11-287 maintained under turbot larval rearing conditions

Permanence and detachment kinetics were different for the three supports tested. In all cases, a release of non-attached bacteria was observed during the first hours.

Porous supports provided better permanence of *Phaeobacter* 27-4 in the biofilters than plastic supports, in which a quick detachment was observed in the first 48 – 72 h. CC biofilters showed the lowest slope of detachment, probably due to the best development of the biofilm as shown by SEM. Slow and continuous detachment in CC biofilters indicates that these biofilters were acting as a reservoir of the probiotic

bacteria, guaranteeing the permanence for a longer period of time and a constant inoculum of the probiotic to seawater.

DGGE profiles showed that bacterial carrying capacity of biofilters and green seawater was low. The introduction of biofilters contributed to diminish the number of bands and thus *Rr* values. Changes in biofilters were marked by the different colonization of the biofilters by *Phaeobacter* and other bacteria. Less pronounced changes were observed in the seawater of the rearing tanks with the different biofilters than in seawater of the *Control* tanks. This result indicates that the introduction of the biofilters in green seawater tanks contributes to stabilize the bacterial microbiota of the system.

CC biofilters showed the lowest number of bands, indicating that *Phaeobacter* would be better established in ceramics than in the other supports and thus would avoid the colonization by other bacteria present in the seawater of the rearing tanks.

Accordingly to all these results, CC biofilters, a more resistant material than sintered glass pellets and thus more suitable for industrial application, were selected for pilot-scale experiments.

In small scale trials conducted under larval rearing conditions, matured *Phaeobacter* 27-4 biofilters antagonized *L. anguillarum*, which disappeared from seawater. *Phaeobacter* released from biofilters to seawater and maintained in constant levels in both seawater and biofilter. High level of nutrients, reproducing high organic matter conditions in rearing tanks, increased the concentration of the probiotic in seawater but did not affect probiotic concentration in the biofilters. The addition of MB to the rearing tanks increased the proportion of *Vibrionaceae* in seawater. This finding is in accordance with the results obtained by Planas *et al.* (2006). Biofilters did not affect total bacteria concentration in the system but reduced *Vibrionaceae* both in seawater and biofilters, even in presence of high concentration of nutrients.

6.4.4. *In vivo* probiotic effect of *Phaeobacter* 27-4 biofilters on turbot larvae

The application of *Phaeobacter* 27-4 biofilters conducted to a decrease in seawater pathogen levels, resulting in a reduction of *L. anguillarum* numbers in larvae

at day nine post-hatching and a reduction of mortalities. But, in spite of the effect observed, the probiotic bacterium was not detected in turbot larvae. These findings confirm that this strain is incapable to colonize turbot larvae (Planas *et al.* 2006).

The use of *Phaeobacter* biofilters significantly reduced mortalities caused by *L. anguillarum*, especially from day eight post-hatching. At day ten, larval mortality attained the same values as the uninfected control (35 – 40%), whereas in infected larvae the mortality was 76%. Planas et al (2006) introduced *Phaeobacter* via rotifers and used the same *L. anguillarum* strain and the same experimental infection model (via rotifer) obtaining mortalities slightly higher at day ten post-hatching. Nevertheless, to compare these two application methods an adequate experimental design using the same batch of larvae, rotifers and microalgae, would be needed.

As demonstrated by Pintado *et al.* (in press), *Phaeobacter* 27-4 does not colonize rotifers permanently but remains inside rotifers a period of time considered sufficient for the larvae to graze on them. In the present study, *Phaeobacter* 27-4 was transferred from biofilters to seawater. The levels of the probiotic in seawater were similar to those observed by Planas *et al.* (2006) in rearing tanks, when the probiotic was bioencapsulated in rotifers and delivered to turbot larvae. In both cases it could be assumed that the probiotic effect may be also occurring from the seawater. However, the effect of *Phaeobacter* 27-4 in seawater would be limited by the permanence of the probiotic in the rearing tanks. When inoculated at a concentration of 10^7 CFU·ml^{-1} in green seawater *Phaeobacter* was not detected after 72 h (Pintado *et al.*, in press). In contrast, the presence of the biofilter maintained a constant concentration of the probiotic in the seawater of tanks, from days seven/eight until the end of the experiment (day ten).

L. anguillarum does not colonize the gut of turbot larvae, but was detected in the epidermis of the larvae (Planas *et al.*, 2005). In this study, it was observed that *L. anguillarum* was released from rotifers to seawater and this could be favouring the preferential infection of the epidermis. Therefore, the prevention of infections by *L. anguillarum* could be more efficient by the control of pathogenic bacteria in seawater. This result demonstrate that the use of biofilters has the advantage of simplifying the procedure to a single application, avoiding the repeated culture and bioencapsulation of the probiotic.

The levels of *L. anguillarum* in seawater were reduced by the introduction of *Phaeobacter* 27-4 biofilters in the rearing tanks. This is in contrast with the results obtained by Planas *et al.* (2006), who did not observe a drop in *L. anguillarum* concentration and hence, attributed the *Phaeobacter* probiotic effect to the reduction in pathogenesis or to the performance of the antagonistic effect at specific sites. This discrepancy in *L. anguillarum* counts could be explained by differences in the techniques used for identification of introduced strains. In the present study, a real-time PCR technique (Prol *et al.*, 2009) was employed to specifically quantify the introduced strains whereas Planas *et al.* (2006) identified both pathogen and probiotic visually from MA and TCBS plates, which could lead to a bias in the identification and quantification of the target strains. However, the existence of different *Phaeobacter* 27-4 mechanisms of action depending on the delivery procedure applied (addition to seawater, bioencapsulated in live prey or immobilized in biofilters) must not be discarded.

Certain *Vibrio* species are considered potentially pathogenic opportunistic bacteria and some have caused infectious diseases in fish farms (Zhang and Austin, 2000). High total *Vibrionaceae* numbers in seawater, live prey or facility surfaces constitutes a potential threat to farmed organisms. *L. anguillarum* and *V. splendidus* are typically related to live prey and able to colonize rotifers and to be released to seawater from rotifers (Prol *et al.*, in press). On the other hand, turbot larval rearing conditions can enhance the proliferation and maintenance of pathogenic bacteria in the system (rotifers and/or seawater), promoting the re-infection of the larvae (Olsson *et al.*, 1998; Sugita *et al.*, 2008). A strategy based on the control of potential pathogenic bacteria, such as *L. anguillarum* and *V. splendidus*, in the seawater of the rearing tanks would be a good option to prevent bacterial infections in larvae. Salvesen *et al.* (1999) produced matured seawater by passing filtered seawater through a biofilter and demonstrated that the use of matured seawater can select for non-opportunistic bacteria having beneficial effects on turbot larvae. Species of the genera *Roseobacter* and *Ruegeria* have been found to be well represented in biofilters and seawater of Recirculating Aquaculture Systems (RAS) but potential pathogens, such as *Vibrio* sp, have been also detected associated with the biofilters in RAS systems (Michaud *et al.*, 2009). In the present study, *Vibrionaceae* bacteria were also detected associated with the biofilters, but at a lower concentration when biofilters were previously colonized with *Phaeobacter*. Results obtained have demonstrated that the introduction of *Phaeobacter* 27-4 biofilters in rearing tanks is an alternative to the spontaneous bacterial colonization (Salvesen *et al.*, 1999), preventing larval infections by reducing total *Vibrionaceae* in seawater and biofilters.

Turbidity has been negatively correlated with growth and survival in red tilapia fry (Ardjosoediro and Ramnarine, 2002). Also, one year old turbots seem to feed better at low turbidity levels (Mallekh *et al.*, 1998). A *Bacillus coagulans* strain (Zhou *et al.*, 2009) and a commercial probiotic (Gomes *et al.*, 2009) exerted a probiotic effect on fish and shellfish by improving seawater quality. Similarly, in this study it has been demonstrated that *Phaeobacter* 27-4 biofilters also contribute to seawater conditioning in the rearing tanks by diminishing turbidity.

6.4.4.1. Influence of *Phaeobacter* 27-4 biofilters on microbial community of turbot larvae

Phaeobacter 27-4 and *L. anguillarum* 90-11-287 were not detected in turbot larvae by DGGE analysis. The absence of a band corresponding to *Phaeobacter* 27-4 in larvae could be due to the inability of this strain to colonize turbot larvae, as mentioned above, even when introduced via rotifers (Planas *et al.*, 2006). On the other hand, the detection limit of DGGE could explain the absence of a band with a migration pattern similar to *L. anguillarum*, as the pathogen concentration in larvae was low (1-10 CFU·larvae^{-1}, accordingly to real-time PCR analysis). DGGE may not be adequate to monitor specifically introduced bacteria, but is a useful tool to detect changes caused by the introduction of probiotic and pathogenic strains in larval rearing systems (Pintado *et al.*, in press; Prol *et al.*, in press; Qi *et al.*, 2009).

The incorporation of *L. anguillarum* or *Phaeobacter* did not displace or modify significantly the bacterial microbiota present in the larvae, whose bacterial diversity was low, as demonstrated by *Rr* values. Little differences between treatments were observed on bacterial microbiota of larvae, which showed similar evolution with time.

Phaeobacter 27-4 and *L. anguillarum* 90-11-287 bioencapsulated in rotifers did not displace or modify rotifers bacterial community (Pintado *et al.*, in press, Prol *et al.*, in press). Samples of rotifers taken from the tanks at the end of the experiment, showed a microbiota more similar to the one from seawater, than to uninfected rotifers enriched with microalgae. This confirms that an exchange of bacteria between rotifers and seawater occurs (Prol *et al.*, in press).

Differently to what was observed in larvae, the presence of *Phaeobacter* 27-4 biofilters conduced to a decrease in the carrying capacity (*Rr*) of seawater in the rearing

tanks, suggesting a direct effect of *Phaeobacter* 27-4 biofilters in seawater bacterial communities.

Marine fish larvae accumulate bacteria by drinking seawater for osmorregulation (Reitan *et al.*, 1998) and by feeding on live prey (Fjellheim *et al.*, 2007; Skjermo and Vadstein, 1993; Verschuere *et al.*, 1997). In this study, changes observed in the bacterial community of turbot larvae reflected the influence of surrounding seawater and rotifers. Bacterial microbiota of larvae was more influenced by the bacteria in rotifers used to feed them than by those in the seawater (Figure 6.10). Similarly, several studies have demonstrated that the intestinal microbiota in first feeding turbot larvae is more dependent on the bacterial community of live prey than on the microbiota present in the seawater (Blanch *et al.*, 1997; Munro *et al.*, 1993; Reitan *et al.*, 1998).

L. anguillarum bioencapsulated in rotifers was released from prey to seawater, where the bacterium reached levels similar to those previously reported by Prol *et al.* (in press). As mentioned, *L. anguillarum* has been reported to infect the epidermis of turbot larvae (Planas *et al.* 2005). Accordingly, it is feasible that larval infection occurs via seawater rather than via rotifers. These findings together with the changes produced by *Phaeobacter* biofilters on bacterial communities of seawater, reducing *Rr* values, seem to indicate that the effect of *Phaeobacter* biofilters would be by acting as a control agent in seawater of the rearing tanks rather than in the larvae themselves.

In the four treatments (*Control*, *BPh*, *La* and *LaBPh*), it was observed over time that 20 % of the bands in DGGE profiles corresponded with an average of 49 % and 64 % of the cumulative intensity of the bands in turbot larvae and seawater, respectively. This means that the most fitting species were dominant and present in high numbers whereas the majority (the remaining 80 % on the x-axis) was present in lower amounts. This internal structure of the highly dynamic larvae bacterial community suggests that only a small group of species played a numerically dominant role at a given moment and that this dominance was even more pronounced for seawater than for larvae.

6.5. Conclusions

Phaeobacter 27-4 grew and antagonized differently the fish pathogens *L. anguillarum* 90-11-287 and *V. splendidus* DMC-1, when immobilized on biofilters made of different materials. The degree of antagonism was affected by the support and

culture conditions of the probiotic strain used. *Phaeobacter* 27-4 ten days matured biofilters prepared with ceramic cylinders guaranteed the permanence of the probiotic bacteria in the rearing tanks for at least eight days, with a transference and maintenance of the probiotic in seawater. Biofilters increased the survival of turbot larvae challenged with *Listonella anguillarum* 90-11-287 by diminishing pathogen levels mainly in seawater and by improving seawater quality.

The antagonistic effect observed both *in vitro* and *in vivo* experiments could be due to the *Phaeobacter* bacteria immobilized in biofilters and/or the ones transferred from biofilters to seawater. To elucidate this point, further work would be needed, with regard the expression of genes related to the antagonistic compound production. In the same way, experiments with a mutant strain without the capability to produce antagonistic compounds should be conducted to analyse separately the possible effect of competition for nutrients and the inhibition.

This is the first time that a probiotic bacterium has been cultured on biofilters and applied *in vivo* in rearing systems. The use of probiotic strains, such as *Phaeobacter* 27-4, in biofilters improves the residence time of the probiont in larval rearing systems when compared to repeated addition protocols (e.g. directly to the seawater or bioencapsulated in rotifers). Biofilters would act as a constant inoculum of the probiotic bacteria to the system, constituting a potentially useful tool in the control of pathogens and opportunistic bacteria in industrial rearing units, both in open and recirculating systems. However, the competition between introduced probiotics and nitrifying bacteria for oxygen, nutrients and space inside the biofilms could reduce nitrification rates in biofilters. Therefore, further research on this aspect should be carried out to ascertain the impact of the introduced probiotic bacteria on the global microbiota of rearing systems.

6.6. References

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7

FINAL DISCUSSION, CONCLUSIONS & FUTURE PERSPECTIVES

7. FINAL DISCUSSION, CONCLUSIONS & FUTURE PERSPECTIVES

7.1. Final discussion

The study of probiotics requires *in vitro* and *in vivo* trials to assay the effectiveness of probiotic strains, to elucidate the mechanisms of action and to select the most adequate application procedure. One of the limitations for the study of probiotics is to track the presence and quantity of the probiotic introduced into the rearing system and, in the case of challenge trials, also of the introduced target pathogenic bacteria. This fact is determinant to evaluate the efficiency and to establish the mechanism of action, which can be difficult to assay by culture dependent methods, due to the associated microbiota and the complex matrix of the samples.

In this Ph.D. Thesis, the first step was to set up a specific and sensitive method for the quantification of the probiont *Phaeobacter* 27-4 and the target pathogenic bacteria, *Listonella anguillarum* 90-11-287 and *Vibrio splendidus* DMC-1, based on real-time PCR. Primers specific for *Phaeobacter* 27-4 were designed within a gene encoding for a lytic transglycosylase (*tdbC*), which is involved in the synthesis of the antagonistic compound tropodithietic acid (TDA) (Geng *et al.*, 2008). For detection and quantification of the pathogens, the specific primers were designed within a gene related to haemolytic activity for *V. splendidus* (Macpherson, 2004) and within the gene *virA/virB*, which encodes for a major surface antigen essential for the virulence of *L. anguillarum* serotype O1 (Norqvist and Wolf-Watz, 1993). This real-time PCR method demonstrated to be accurate and reliable and was used in this Ph.D. Thesis to quantify the introduced bacteria in seawater, biofilters and all the organisms involved in turbot larval rearing (microalgae, rotifer, *Artemia* and turbot larvae), both in *in vitro* and *in vivo* trials.

When studying a new strategy for the control of infections, it is essential to know the way pathogens get into the rearing system. It is well known that live prey (rotifers and *Artemia*) are the principal entry route of bacteria in fish rearing systems (Munro *et al.*, 1993). Rotifer is commonly used as the first live prey in the food chain of turbot larvae. Some opportunistic pathogenic bacteria, such as *Vibrionaceae*, enter into the system via rotifer and cause mortalities in fish larvae (Reid *et al.*, 2009; Sandlund and

Bergh, 2008; Thomson *et al.*, 2005; Verdonck *et al.*, 1997). Understanding the colonization and permanence of pathogenic *Vibrionaceae* bacteria in live prey is a fundamental step to understand the mode fish larvae get infected and to develop effective prevention strategies against those bacteria.

In this Ph.D. Thesis, it has been demonstrated that the pathogens *L. anguillarum* 90-11-287 and *V. splendidus* DMC-1, which are known to cause mortalities in turbot larvae when introduced via rotifers in challenge trials (Planas *et al.*, 2005; Thomson *et al.*, 2005), differ in their capabilities to colonize and maintain in the rotifer *Brachionus plicatilis*.

The higher colonization and residence time in rotifers of *L. anguillarum* and the higher release from rotifers to seawater of *V. splendidus* could determine different infection patterns in both pathogens. Accordingly, it would be expected that the infection of larvae by *L. anguillarum* mainly occur by ingestion of rotifers and subsequent colonization of the gut, whereas the infection with *V. splendidus* would be favoured by an active intake of seawater (gut colonization) or by direct contact with surrounding seawater (epidermis colonization). However, these assumptions do not agree with previous findings. In a challenge trial carried out with turbot larvae fed on rotifers infected with *L. anguillarum* 90-11-287, Planas *et al.* (2005) observed that the pathogen did not colonize the intestine but affected the epidermis of larvae, suggesting that the infection might occur after the release of the pathogen from rotifers to seawater and subsequent colonization of epidermic tissues (Pérez-Lorenzo *et al.*, 2006). On the other hand, *V. splendidus* DMC-1 has been detected as the main species in the gut of diseased turbot and cod larvae (Reid *et al.*, 2009; Thomson *et al.*, 2005), which would suggest that ingestion of infected rotifers would be the main way of entry of this pathogen into the larvae. Other factors, such as the adhesion capability to mucus, might be also relevant as it has been demonstrated that the potential of *Vibrionaceae* bacteria to adhere to different types of mucus in different fishes is strain dependent (Balebona *et al.*, 1995; Bordas *et al.*, 1998) and should be investigated.

DGGE analysis of samples from the rotifer infection trials pointed out that the rotifers microbial community did not present marked dominance of bacterial species. Bacterial community in rotifers was composed by groups previously found in hatcheries of different fish species: α-Proteobacteria, γ-Proteobacteria and CFB (Griffiths *et al.*, 2001; McIntosh *et al.* 2008; Sandaa *et al.*, 2003; Schulze *et al.*, 2006), as well as by the Gram positive bacterium *Microbacterium* sp, probably associated with the cultures of

the microalgae *I. galbana* (Makridis *et al.*, 2006). Fluctuations observed in rotifers bacterial community could be explained by a bacterial exchange between rotifers and seawater. This fact would explain the rapid temporary colonization and the subsequent release to seawater of the pathogenic *Vibrionaceae*. Therefore, strategies directed towards bacterial control in seawater of live prey cultures and/or rearing tanks would be an efficient strategy for prevention of infections caused by *Vibrionaceae* bacteria, as *L. anguillarum* and *V. splendidus*.

In a previous work, Planas *et al.* (2006) demonstrated the *in vivo* probiotic effect of *Phaeobacter* 27-4, increasing the survival of turbot larvae experimentally infected with *L. anguillarum*, when the probiotic was delivered bioencapsulated in rotifers (Planas *et al.*, 2006). However, *Phaeobacter* 27-4 did not colonize larval gut or rotifers, being its permanence in the seawater of rearing tanks limited (Pintado *et al.*, in press; Planas *et al.*, 2006). This constraint made necessary the application of repeated additions of the probiotic in the larvae rearing tanks to assure the permanence and the effect of the probiotic in the rearing system.

Similarly to other Roseobacters, *Phaeobacter* 27-4 has the capability to attach to surfaces and subsequently form a biofilm composed by rosette-shaped microcolonies (Bruhn *et al.*, 2006), being the production of the antagonistic compound TDA related to biofilm formation (Bruhn *et al.*, 2005, 2007).

In the present Ph.D. Thesis, a new strategy based on the immobilization of the probiotic strain *Phaeobacter* 27-4 in biofilters was studied. This new strategy guarantees the long-term permanence of probiotic bacteria in the rearing tanks, favouring the protection of fish larvae against pathogenic *Vibrionaceae* strains.

The growth of the probiotic was tested with different supports used in aquaculture biofilters (plastic balls, sintered glass pellets and ceramic cylinders) and under different bacterial culture conditions (soaked stagnant and submerged with or without agitation). *Phaeobacter* 27-4 attached and grew differently depending on the type of material used as biofilter support and on the culture conditions. Bacterial concentration in biofilters along the experiment, which was similar in all supports, did not correlate with the attachment observed by SEM, indicating that bacteria could be also growing in the liquid media inside the interstices of supports. When compared with plastic and sintered glass supports, the higher surface:volume ratio and the higher roughness of ceramic supports, could enhance the formation of a biofilm composed by rosette-shaped

microcolonies, observed when cultured under submerged stagnant conditions. It has been demonstrated by Bruhn *et al.* (2005) that stagnant conditions promote rosettes formation and the development of a biofilm by *Phaeobacter* 27-4 and this finding is in agreement with the results achieved in this Ph.D. thesis.

In vitro inactivation and inhibition of growth caused by *Phaeobacter* biofilters on *L. anguillarum* 90-11-287 and *V. splendidus* DMC-1 were evaluated. In absence of nutrients, inactivation was similar in both pathogens but the inhibition of growth promoted by the probiotic in presence of nutrients was higher in *L. anguillarum* than in *V. splendidus*. In presence of *Phaeobacter*, *V. splendidus* grew more than *L. anguillarum* and induced a lower growth of the probiotic in the culture media. These results are explained by the different maximum specific growth rate of the probiotic and the two pathogens used, which is higher for *V. splendidus* than for the other two strains (Vázquez *et al.*, 2004), suggesting the existence of nutrients competition between the probiotic and the pathogens.

In the inactivation trial (no nutrients added), the level of *Phaeobacter* released from biofilters to seawater was similar in all cases, suggesting that the effect could be also exerted by the probiotic bacteria present in the seawater. In the inhibition trial (nutrients added), the concentration of *Phaeobacter* in the medium was also similar in all treatments, indicating that pathogens inhibition would be directly dependent on the probiotic present in the biofilters.

The presence of *Phaeobacter* diminished pathogens attachment to ceramic and sintered glass but not to plastic support. This result is in accordance with those obtained by Rao *et al.* (2006) who found that *Phaeobacter gallaeciensis* strain 2.10 is able to invade and disperse pre-established biofilms on algae.

Permanence and detachment kinetics of *Phaeobacter* from the three supports were tested in tanks with green seawater maintained under the conditions used in larval rearing. A better permanence was observed in porous supports, compared with the plastic one, being the lowest slope of detachment registered in ceramic biofilters, probably due to the formation of the well developed biofilm. Additionally, in DGGE profiles, ceramic biofilters showed the lowest number of bands, indicating that the presence of *Phaeobacter* in these biofilters avoided the colonization by other bacteria.

In the *in vivo* trials carried out at pilot-scale, ceramic cylinders were selected as the best support for preparation of the biofilters due to the capability of *Phaeobacter* 27-4 in that support to: *i*) develop a biofilm formed by rosette-shaped microcolonies, *ii*) antagonize two pathogens (*L. anguillarum* and *V. splendidus*), and *iii*) remain longer in the biofilters when maintained under larval rearing conditions. Ceramic supports were also selected due to its higher resistance, which would be advantageous for the scale-up in aquaculture facilities.

In small scale trials conducted under larval rearing conditions and different nutrients levels, matured biofilters with *Phaeobacter* 27-4 antagonized *L. anguillarum* in the seawater of rearing tanks, until the pathogen was undetectable. The presence of the probiotic also reduced the levels of total *Vibrionaceae* both in seawater and biofilters, even in presence of high concentration of nutrients.

The *in vivo* probiotic effect of *Phaeobacter* 27-4 immobilized in the biofilter was demonstrated in challenge trials where the larvae were experimentally infected with *L. anguillarum* via rotifers. The presence of *Phaeobacter* 27-4 matured biofilters significantly reduced the mortalities caused by the pathogen, by diminishing the concentration of *L. anguillarum* mainly in seawater. In spite of this effect, *Phaeobacter* was not detected in larvae, which confirms the incapacity of this bacterium to colonize turbot larvae (Planas *et al.*, 2006).

DGGE profiles in larvae and seawater from the challenge trials revealed that the introduction of the probiotic and/or the pathogen did not displace or modify significantly the bacterial microbiota present in larvae, characterized by their low bacterial diversity. However, the presence of *Phaeobacter* 27-4 biofilters diminished the bacterial carrying capacity in seawater of rearing tanks, indicating an inhibitory effect against the bacteria present in the seawater of turbot larvae rearing tanks.

The analysis of the bacterial community of rotifers taken from the tanks at the end of the *in vivo* trial showed a higher similarity with the bacterial microbiota in seawater at the same time than with the one of rotifers analysed at the moment of their addition to the tanks. This finding suggests the existence of bacterial exchange between rotifers and seawater (Prol *et al.*, in press). On the contrary, bacteria of larvae seemed to be more influenced by the bacteria present in the prey (rotifers) used to feed them than by the bacterial microbiota of seawater. Hence, controlling potentially pathogenic or opportunistic bacteria in seawater would avoid their transference to rotifer and

subsequently to larvae. As mentioned above, *L. anguillarum* was found to be mainly associated with the epidermis of turbot larvae infected via rotifers and also in the seawater of rearing tanks containing infected larvae (Planas *et al.*, 2005). In this sense, larval infection very likely occurs via seawater rather than via rotifers. Changes produced by *Phaeobacter* biofilters on seawater bacterial community indicate their effect acting as a control agent of *Vibrionaceae* in the seawater of the rearing tanks rather than in the larvae themselves.

In addition to the antagonistic effect, diminishing total *Vibrionaceae* concentration, the probiotic biofilter contributed to the increase of seawater quality in the rearing tanks by reducing its turbidity.

Biofilters acted as a probiotic reservoir in the rearing system, maintaining the level of the probiotic in the seawater of tanks for at least ten days, period in which larvae are fed on rotifers and which is considered critical for larval survival. An important advantage of this biofilters is that their use does not require repeated additions of the probiotic, avoiding long-term cultures and bioencapsulation in rotifers. This feature simplifies the potential transference of this methodology to industrial hatcheries.

7.2. Conclusions

The following conclusions could be established from the results achieved in each chapter of this Ph.D. Thesis:

1. **Chapter I**. Real-time PCR detection and quantification of the fish probiotic *Phaeobacter* strain 27-4 and two fish pathogenic *Vibrionaceae* in microalgae, rotifer, *Artemia* and first feeding turbot (*Psetta maxima*) larvae.

    1.1. The developed real-time PCR protocol is reproducible and specific.

    1.2. The method requires separate standard curves for each host organism.

    1.3. The optimized real-time PCR method can be used to detect and quantify the probiotic *Phaeobacter* 27-4 and the pathogens *L. anguillarum* 90-11-287 and *Vibrio splendidus* DMC-1 bioencapsulated in rotifers and introduced in turbot larvae.

1.4. The method allows monitoring and quantification of *Phaeobacter* 27-4, *L. anguillarum* 90-11-287 and *Vibrio splendidus* DMC-1 in seawater, rotifers and turbot larvae during *in vivo* trials and is considered a useful tool for detecting these bacteria in industrial rearing units.

2. **Chapter II.** Different colonization and residence time of *Listonella anguillarum* and *Vibrio splendidus* in the rotifer *Brachionus plicatilis* determined by real-time PCR and DGGE.

2.1. *L. anguillarum* 90-11-287 and *V. splendidus* DMC-1, when introduced via rotifers in the larvae rearing tanks, are able to remain in rotifers and seawater enough time to infect fish larvae.

2.2. The performance of both pathogenic strains is different as colonization and residence time of *V. splendidus* in the rotifer *Brachionus plicatilis* were lower than for *L. anguillarum*, being released faster to surrounding seawater.

2.3. The bacterial community of rotifers did not present marked species dominance and was composed by Gram negative bacteria belonging to α-Proteobacteria, γ-Proteobacteria, *Cytophaga-Flexibacter-Bacteroides* group and a Gram positive bacterium (*Microbacterium* sp).

2.4. The incorporation of *L. anguillarum* or *V. splendidus* did not reduce bacterial diversity and shifts in bacteria populations could be explained by bacterial exchange between rotifers and seawater.

3. **Chapter III**. Application of biofilters with the probiotic strain *Phaeobacter* 27-4 against *Vibrionaceae* infections in the rearing of turbot (*Psetta maxima*) larvae.

3.1. Selection of the biofilter support and the culture conditions for *Phaeobacter* 27-4:

3.1.1. *Phaeobacter* 27-4 attached and grew differently depending on the type of material used as biofilter support (plastic, sintered glass or ceramics) and on the culture conditions (soaked and submerged with or without agitation).

3.1.2. *Phaeobacter* 27-4 formed a multilayer biofilm with the characteristic rosette-shaped microcolonies only on ceramic cylinders cultured in submerged stagnant conditions.

3.1.3. *Phaeobacter* 27-4 antagonized differently the fish pathogens *L. anguillarum* 90-11-287 and *V. splendidus* DMC-1 depending on the materials in which it was immobilized, as well as on the pathogen tested.

3.1.4. In absence of nutrients, the inactivation was similar for both pathogens, but the inhibition of growth promoted by the probiotic, in presence of nutrients, was higher for *L. anguillarum* than for *V. splendidus*. The presence of *Phaeobacter* diminished the attachment of both pathogens to ceramic and sintered glass but not to plastic supports.

3.1.5. The permanence of *Phaeobacter* 27-4 in biofilters prepared with ceramic supports, after transference to green seawater tanks and maintenance under turbot larval rearing conditions, was higher than in biofilters prepared with plastic or sintered glass and guaranteed the permanence of probiotic cells in the rearing tanks for at least eleven days.

3.1.6. Ceramic cylinders were selected due to the capability of *Phaoebacter* 27-4 to *i*) develop a biofilm formed by rosette-shaped microcolonies, *ii*) antagonize two pathogens (*L. anguillarum* and *V. splendidus*), and *iii*) remain longer in the biofilters when maintained under larval rearing conditions. Additionally, ceramics is a resistant material, which would be advantageous for the scale-up in aquaculture facilities.

3.2. Antagonism of matured *Phaeobacter* 27-4 ceramic biofilters, in pilot-scale trials, conducted under turbot rearing conditions:

3.2.1. *Phaeobacter* 27-4 biofilters reduced the concentration of *L. anguillarum* 90-11-287 and total *Vibrionaceae* in green seawater with different nutrients levels.

3.2.2. *Phaeobacter* 27-4 biofilters significantly reduced accumulated mortality in turbot larvae infected with the pathogen *L. anguillarum* 90-11-287 by diminishing pathogen levels and the concentration of total *Vibrionaceae*, mainly in seawater. Additionally, the probiotic biofilter reduced turbidity in the rearing tanks.

3.3. DGGE analysis of bacterial microbiota in turbot larvae, rotifer and seawater in the *in vivo* challenge trial, revealed that:

3.3.1. The incorporation of *L. anguillarum* via rotifer or *Phaeobacter* 27-4 in the matured biofilters did not displace or modify significantly the bacterial microbiota in larvae, which showed low bacterial diversity.

3.3.2. The presence of *Phaeobacter* 27-4 biofilters diminished the bacterial carrying capacity in seawater of rearing tanks, indicating a direct effect of the probiotic biofilters in seawater bacterial communities.

3.3.3. The bacterial community of turbot larvae was more influenced by bacteria present in the rotifers used to feed them than by the bacterial microbiota in seawater.

3.3.4. The bacteria of the seawater of rearing tanks had influence on bacterial microbiota of rotifers.

3.3.5. The biofilters with *Phaeobacter* 27-4 acted as a control agent of *Vibrionaceae* in the seawater of larvae rearing tanks rather than in the larvae themselves.

7.3. Future perspectives

Results obtained in the present Ph.D. Thesis comply with the proposed objectives. However, new subjects of research are opened from the summarized conclusions.

An important aspect that should be considered in the future is to identify whether the *Phaeobacter* 27-4 antagonistic effect is caused by the bacteria immobilized on the biofilters or to those transferred from biofilters to surrounding medium (or to both). To elucidate this point, real-time PCR quantification of the expression of genes related to

TDA production could be a useful tool. In the same way, experiments with a mutant strain without the capability to produce antagonistic compounds would permit to analyse separately the possible effect of competition for nutrients and the inhibition.

Bacterial infections in fish larvae are mainly caused by opportunistic bacteria and therefore, the protection of fish larvae would require the use of probiotics with a wide-ranging antagonistic activity. *Phaeobacter* 27-4 antagonizes bacteria from *Vibrionaceae* and CFB group. The effect against pathogens with different infection patterns, such as *V. splendidus* or *Tenacibaculum* spp, should be determined by *in vitro* and *in vivo* trials. Also, other taxonomical groups should be tested for antagonism. A comparative study with biofilters colonized by other antagonistic and biofilm-forming bacteria of the *Roseobacter* clade, such as *Phaeobacter gallaeciensis*, *P. inhibens*, *Ruegeria mobilis* and *R. pelagia*, would be of interest (Brinkhoff *et al.*, 2004; Porsby *et al.*, 2008; Ruíz-Ponte *et al.*, 1998).

The results of this Ph.D. thesis suggest that probiotic biofilters could be also used for the control of bacterial infections by their application in the production of live prey. So, the effect of probiotic biofilters on microalgae, rotifer or *Artemia* cultures should be studied. Probiotic biofilters could be also tested in other fish larvae species. In the case of larval rearing using recirculating systems, antagonism or competition between the introduced bacteria and heterotrophic or nitrifying bacteria in the biofilter could occur. Therefore, further research on this aspect should be carried out to ascertain the impact of the introduced probiotic bacteria on the global microbiota of rearing systems.

The final step would be to test *Phaeobacter* 27-4 biofilters in marine fish rearing industrial plants. Additionally, this transfer would require: a) an analysis of current legislation and constrains, b) an economical assessment for the production of probiotic biofilters suitable to aquaculture industry and c) a cost-benefit study of the implementation, which would determine the profitability of probiotic biofilters applied to industrial scale.

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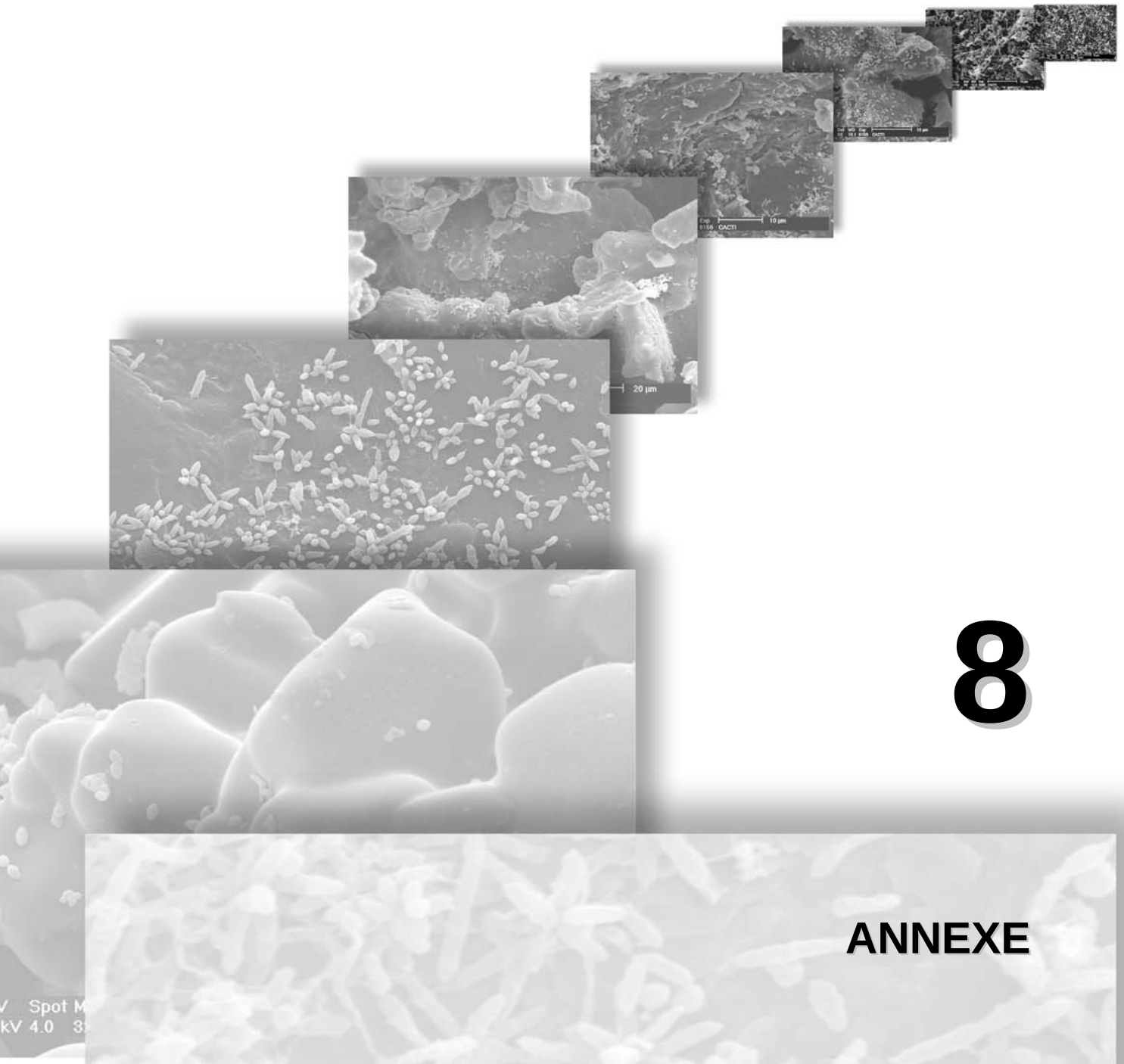
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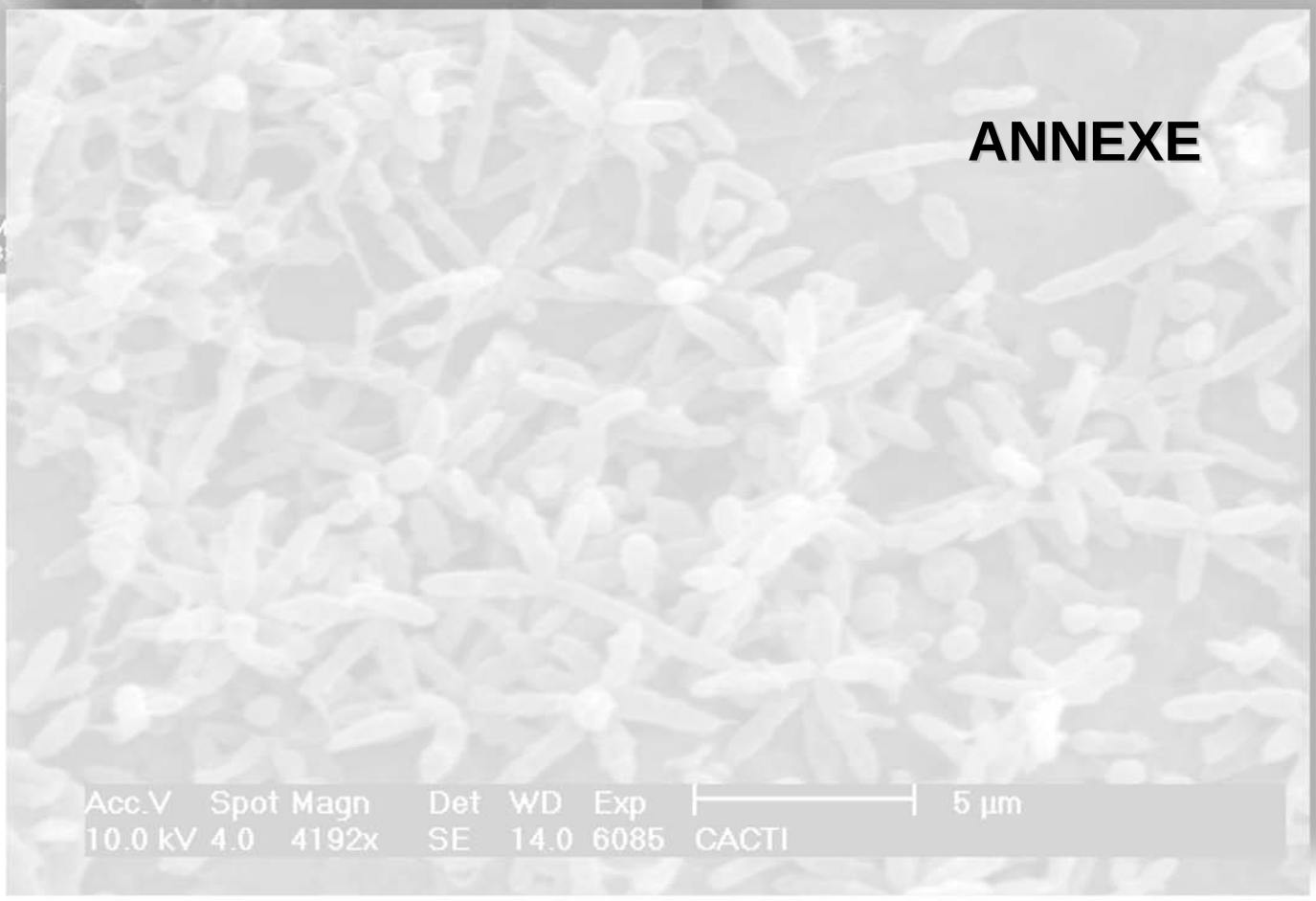
ANNEXE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8. RESUMO EN GALEGO (*ABSTRACT IN GALICIAN*)

Inmobilización de *Phaeobacter* 27-4 en biofiltros como unha estratexia fronte a infeccións causadas por bacterias da familia *Vibrionaceae* no cultivo larvario de peixes

8.1. Introdución

Un dos factores que está a limitar a expansión da acuicultura de peixes mariños é o subministro de xuvenís. A produción intensiva de larvas de peixes soe ir ligada á proliferación de bacterias, as que poden inducir a un crecemento pobre ou a unha mortalidade masiva das larvas. Na meirande parte dos casos, a mortalidade larvaria soe ocorrer como consecuencia da proliferación de bacterias oportunistas, máis que como consecuencia dun patóxeno específico (Olafsen 1993, 2001). As altas densidades larvarias e os altos niveis de materia orgánica (feces, larvas mortas ou presas vivas) presentes nos sistemas de cultivo larvario fan destes sistemas un entorno propicio para a proliferación bacteriana. O control bacteriano nos sistemas de cultivo larvario e na produción de presas vivas é un factor determinante para a supervivencia larvaria (Dhert *et al.*, 2001; Planas e Cunha, 1999; Skjermo e Vadstein, 1999), o que está demostrado polo incremento da supervivencia de larvas tratadas con antibióticos (Gatesoupe, 1982, 1989; Pérez-Benavente e Gatesoupe, 1988).

O emprego de antibióticos no medio acuático leva ó desenvolvemento de resistencias, podendo transferirse a outras bacterias como patóxenos de peixes ou incluso do ser humano (Cabello, 2006). Deste xeito o emprego dos antibióticos debería manterse reducido á mínima expresión. O emprego de axentes quimioterapéuticos na acuicultura viuse reducido grazas ás vacinas, as que incrementaron a supervivencia do peixe cultivado (Lunestad, 1998; Subasinghe, 1997; Verschuere *et al.*, 2000). Non obstante, as larvas de peixe non teñen un sistema inmune desenvolto, estando protexidos basicamente polos anticorpos maternos e unha resposta inmune non específica (Vadstein, 1997).

A meirande parte dos protocolos empregados para controlar o crecemento bacteriano no cultivo intensivo de larvas de peixes mariños teñen como finalidade o desenvolvemento dun ambiente limpo mediante o tratamento da auga (p ex. filtración, irradiación con luz UV, ozonización, uso de desinfectantes) (Salvesen e Vadstein, 1995). As medidas de control bacteriano nas presas vivas baseados na desinfección dos ovos de rotífero para a produción de cultivos axénicos (Dhert *et al.*, 2001; Douillet, 1998), o tratamento con peróxido de hidróxeno (Giménez *et al.*, 2006) ou o tratamento con luz UV para a descontaminación parcial (Munro *et al.*, 1993b, 1999) tamén poderían ser ferramentas útiles. Non obstante, a eliminación completa das bacterias nos organismos e no sistema de cultivo non é posible e a desinfección implica, na meirande parte dos casos, a perda da poboación microbiana estable composta principalmente por bacterias de crecemento lento (estrategos do *K*), promovendo, nun ambiente rico en nutrientes (como consecuencia da comida e das excrecións) como son os sistemas de cultivo larvario, a rápida colonización por bacterias oportunistas (estrategos do *r*), con altas taxas de crecemento e potencialmente daniñas para as larvas de peixe (Skjermo e Vadstein, 1993). Polo tanto, na actualidade, os esforzos encamíñanse cara novas estratexias para o control da microbiota bacteriana nos sistemas de cultivo larvario que conducirían a unha acuicultura sostible, evitando o emprego de antibióticos e desinfectantes. Estas estratexias cumprirían coas medidas ecolóxicas para un crecemento e expansión sostibles da acuicultura promovidas pola FAO no "Estado das pesquerías mundiais e da acuicultura" (*The State of the World Fisheries and Aquaculture*, FAO 2006, pp. 76-84).

Os probióticos definidos pola FAO/WHO (2001) como "organismos vivos que, cando se administran nas cantidades adecuadas, confiren un beneficio na saúde do hospedador", constitúen unha ferramenta potencial na redución das mortalidades no cultivo de organismos acuáticos (Gatesoupe, 1999; Gram e Ringø, 2005; Verschuere *et al.*, 2000; Vine *et al.*, 2006). Nos sistemas de acuicultura, onde o hospedador e os microorganismos comparten o mesmo medio, a definición de probiótico foi ampliado por Verschuere *et al.* (2000) para incluír tamén ós microorganismos que poden ter un efecto positivo sen necesidade de colonizar ó hospedador (tracto dixestivo, branquias ou pel) simplemente coa súa presenza na auga. Este abre o concepto para incluír microorganismos que actúan sobre a calidade da auga ou como axentes de biocontrol na mesma.

Os diferentes modos de acción dos probióticos inclúen: produción de compostos inhibidores (antibióticos, bacteriocinas, ácidos orgánicos, peróxido de hidróxeno),

competencia por nutrientes limitantes (sideróforos), contribución enzimática á dixestibilidade da comida, competencia pola adhesión ó mucus, estimulación do sistema inmune ou mellora da calidade da auga. Polo momento e para a meirande parte dos probióticos descoñécese o mecanismo de acción concreto, precisándose nalgúns casos unha combinación de varios mecanismos, p ex. a unión e crecemento no mucus pode ser un requisito previo para exercer a actividade antagonista ou inmunoestimular ó hospedador (Austin e Brunt, 2009). A meirande parte dos traballos publicados sobre probióticos en larvicultura son de investigación aplicada, con poucas evidencias sobre o mecanismo de acción preciso (Vine *et al.*, 2006). Ademais, moitos dos mecanismos probióticos estúdanse *in vitro* o que non garante un efecto *in vivo* (Gram *et al.*, 2001).

O emprego de probióticos sería adecuado nos primeiros períodos do cultivo larvario de xeito que as bacterias que colonizan ós ovos ou ás larvas durante os primeiros períodos antes da alimentación esóxena poderían previr a colonización por parte de bacterias oportunistas procedentes das presas vivas (Carnevali *et al.*, 2004; Hansen e Olafsen, 1999; Olafsen, 2001). Outra vantaxe sería que, a diferenza dos adultos, nas larvas o pH intestinal é alcalino, de xeito que os probióticos potenciais non terían que ser resistentes á acidez (Rønnestad *et al.*, 2000; Tanaka *et al.*, 1996).

Probióticos dispoñibles comercialmente ou bacterias do ácido láctico son os principais microorganismos empregados no cultivo de larvas de peixes (Arndt e Wagner, 2007; Benetti *et al.*, 2008; Carnevali *et al.*, 2004; Gatesoupe, 1991, 1994; Planas *et al.*, 2004; Suzer *et al.*, 2008; Villamil *et al.*, 2003) aínda que nos últimos anos fermentos comerciais tamén se están propoñendo como probióticos (Tovar-Ramírez *et al.*, 2004). Unha mellor estratexia, que evitaría a introdución de microorganismos exóticos no sistema, é seleccionar ós candidatos probióticos entre os illados de peixes sans (Olsson *et al.*, 1992; Ottesen e Olafsen, 2000; Westerdahl *et al.*, 1991) ou das instalacións dos sistemas de cultivo larvario (Hjelm *et al.*, 2004a, b; Huys *et al.*, 2001) xa que a eficacia dos probióticos parece ser máis alta nas especies das que foron illadas (Verschuere *et al.*, 2000).

Xeralmente, a selección dos probióticos está baseada no efecto antagonista sobre bacterias patóxenas responsables de altas mortalidades en larvas, como *Listonella anguillarum* (Olsson *et al.* 1992; Planas *et al.* 2006), *Vibrio* sp (Gatesoupe, 1994; Vine *et al.*, 2004a, b) ou *Aeromonas* sp (Ringø e Vadstein, 1998; Vine, 2004a, b), aínda que outros criterios como a adhesión ó mucus, inmunoestimulación ou incremento da actividade enzimática tamén se están a empregar como criterios de selección

(Gatesoupe, 1994; Suzer *et al.*, 2008). Os probióticos poden engadirse directamente á auga ou introducirse no sistema bioencapsulados nas presas vivas.

8.1.1. *Phaeobacter* 27-4 como probiótico fronte a infeccións causadas por bacterias da familia *Vibrionaceae* en larvas de rodaballo

Membros do grupo *Roseobacter* (α-Proteobacteria) (Brinkhoff *et al.*, 2008), un grupo amplamente distribuído en diferentes ambientes mariños (Buchan *et al.*, 2005; Wagner-Döbler e Biebl, 2006), inclúe varias cepas bacterianas con actividade antagonista fronte diferentes grupos taxonómicos bacterianos mediante a produción de metabolitos secundarios (Brinkhoff *et al.*, 2004; Grossart *et al.*, 2004; Hjelm *et al.*, 2004a, b; Porsby *et al.*, 2008). Esta vantaxe competitiva das Roseobacters levou a estudar a súa aplicación como probióticos na acuicultura (Hjelm *et al.*, 2004a, b; Planas *et al.*, 2006) ou como axentes "*antifouling*" nos sistemas mariños (Egan *et al.*, 2008; Rao *et al.*, 2006).

Algunhas cepas dos xéneros *Phaeobacter* (antigamente *Roseobacter*, Martens *et al.*, 2006) e *Ruegeria*, teñen a capacidade de producir ácido tropodithiético e/ou thiotropocina (Brinkhoff *et al.*, 2004; Bruhn *et al.*, 2005; Porsby *et al.*, 2008). Está demostrado que estes compostos son os causantes da inhibición de diferentes bacterias (Brinkhoff *et al.*, 2004), incluíndo algunhas vibrionaceas patóxenas de peixes (Bruhn *et al.*, 2005; Hjelm *et al.*, 2004a, b; Porsby *et al.*, 2008). A adición de cultivos ou extractos bacterianos de cepas antagonistas de *Phaeobacter gallaeciensis*, *Phaeobacter* spp (Balcázar *et al.*, 2007; Planas *et al.*, 2006; Ruíz-Ponte *et al.*, 1999) ou *Ruegeria* sp (Makridis *et al.*, 2005) nos tanques de cultivo larvario produciron un efecto beneficioso en larvas de peixes (Makridis *et al.*, 2005; Planas *et al.*, 2006) e mariscos (Balcázar *et al.*, 2007; Ruíz-Ponte *et al.*, 1999).

A cepa *Phaeobacter* 27-4 foi illada dunha planta de cultivo de rodaballo en Galicia (Stolt Sea Farm, Merexo, costa Noroeste, España) e identificada mediante secuenciación do 16S do ADNr, como un 99 % semellante a *Phaeobacter gallaeciensis* (Hjelm *et al.*, 2004b). Esta cepa antagoniza a diferentes patóxenos de peixes da familia *Vibrionaceae*, tales como *Listonella anguillarum* e *Vibrio splendidus* (Hjelm *et al.*, 2004a, b), ademais dunha cepa patóxena do xénero *Tenacibaculum* dentro do grupo *Cytophaga-Flexibacter-Bacteroides* que foi illada de rodaballos enfermos (datos non publidados).

Phaeobacter 27-4 é inocua para as larvas de rodaballo, para os rotíferos empregados para a súa alimentación (Pérez-Lorenzo, 2006; Planas *et al.*, 2006), e para a microalga *Isochrysis galbana* (datos non publicados), a que se emprega para o enriquecemento das presas vivas. Esta cepa de *Phaeobacter* incrementou a supervivencia de larvas de rodaballo experimentalmente infectadas con *L. anguillarum* cando se administrou bioencpasulada en rotíferos (Planas *et al.*, 2006). Non obstante, 27-4 non colonizou o tracto dixestivo das larvas ou ós rotíferos, estando a súa permanencia na auga dos tanques larvarios limitada (Pérez-Lorenzo, 2006; Planas *et al.*, 2006). Este tempo de residencia limitado implicou adicións repetidas do probiótico ós cultivos larvarios.

Phaeobacter 27-4 non forma parte da microbiota normal das larvas e este feito podería explicar a súa incapacidade para colonizar ás larvas de rodaballo (Hjelm *et al.*, 2004a). Nun estudo levado a cabo no período dun ano en dúas plantas de rodaballo en Galicia (noroeste de Epaña), o xénero *Phaeobacter* foi predominante entre os illados que amosaron antagonismo fronte a patóxenos da familia *Vibrionaceae*. A meirande parte das Roseobacters antagonistas foron illadas das paredes dos tanques e soamente pequenos números foron detectados na auga dos tanques e en larvas alimentadas con rotíferos e *Artemia* (Hjelm *et al.*, 2004a).

Os membros mariños do grupo *Roseobacter* teñen a habilidade de colonizar rapidamente superficies inorgánicas e orgánicas, nas que se inclúen algas mariñas e dinoflaxelados (Dang e Lovell, 2002; Mayali *et al.*, 2008), sendo considerados como colonizadores primarios de superficies (Dang *et al.*, 2008). A actividade antagonista das Roseobacters parece estar asociado coa súa capacidade para medrar sobre superficies (Slightom e Buchan, 2009), mostrando aquelas bacterias asociadas a partículas unha actividade antagonista meirande cas que viven libres no mar (Long e Azam, 2001). Por outra banda, a produción de moléculas de Quorum Sensing (QS) por diferentes microorganismos relacionouse coa capacidade de colonización de superficies a coa produción de metabolitos secundarios (Davies *et al.*, 1998; Egan *et al.*, 2008; Labatte *et al.*, 2007). Estudos recentes, suxiren que as Roseobacters poderían ser os principais produtores mariños das moléculas de QS chamadas lactonas de homoserina acetiladas (-(AHL, do inglés, *acyl homoserine lactones*) no medio mariño (Cicirelli *et al.*, 2008).

Phaeobacter 27-4 ten a capacidade de producir moléculas AHL (Bruhn *et al.*, 2005), adherirse a superficies inertes e, en consecuencia, formar unha biopelícula composta por microcolonias con forma de roseta (Bruhn *et al.*, 2006). A produción do

composto antagonista, ácido tropodithiético, por *Phaeobacter* 27-4 está relacionado coa produción dun pigmento pardo-amarelado e coa formación de biopelículas na interface aire-liquido (Bruhn *et al.*, 2005, 2007). As condicións de cultivo desta cepa inflúen na produción de moléculas AHL, na formación de rosetas e en consecuencia, na formación de biopelículas, ademais de na produción do composto antagonista. Todos estes aspectos vense favorecidos polo cultivo da bacteria en condicións estáticas (Bruhn *et al.*, 2005, 2006, 2007).

O conxunto destes resultados, suxire que as bacterias formadoras de biopelículas con actividade antagonista, tales como *Phaeobacter* 27-4, poderían empregarse como probióticos mediante a súa inmobilización en biofiltros. Este reduciría o nivel de bacterias patóxenas (Kennedy *et al.*, 1998) ou de bacterias totais (Makridis *et al.*, 2001) na auga, nas presas vivas ou nas larvas de peixes. Estes biofiltros incrementarían a permanencia das bacterias probióticas nos sistemas de cultivo larvario, tanto en sistemas abertos como en recirculación, protexendo ás larvas fronte a bacterias oportunistas ou potencialmente patóxenas como as vibrionaceas.

8.2. Obxectivos

O obxectivo principal desta Tese de Doutoramento foi estudar a inmobilización da bacteria antagonista e formadora de biopelículas *Phaeobacter* 27-4 en biofiltros e demostrar a súa efectividade para o control de infeccións causadas por bacterias da familia *Vibrionaceae* no cultivo larvario de peixes.

Os biofiltros colonizados por bacterias formadoras de biopelículas con actividade antagonista poderían exercer un efecto probiótico nas larvas de peixes mediante a redución das bacterias totais ou patóxenas na auga, presas vivas (rotíferos, *Artemia*) ou nas larvas. Este novo procedemento de aplicación garantiría a permanencia do probiótico no sistema larvario ó tempo que protexe ás larvas de rodaballo (*Psetta maxima*) fronte ás infeccións bacterianas causadas por patóxenos da familia *Vibrionaceae* e incrementa a supervivencia larvaria ó principio da alimentación esóxena.

Para acadar este obxectivo, desenvolveuse un método de PCR a tempo real para detectar e cuantificar especificamente tanto ó probiótico como ós patóxenos diana nos sistemas de cultivo larvario (Capítulo I). Os rotíferos soen ser a vía de entrada nas larvas

para os patóxenos da familia *Vibrionaceae* e, en consecuencia, en esta Tese de Doutoramento, estudouse a colonización e o tempo de residencia dos patóxenos *L. anguillarum* 90-11-287 e *V. splendidus* DMC-1 nos rotíferos (Capítulo II). Tamén se avaliaron o crecemento do probiótico *Phaeobacter* 27-4 en diferentes soportes, o seu tempo de residencia, a actividade antagonista *in vitro* e o efecto *in vivo* do probiótico inmobilizado en biofiltros sobre larvas infectadas cun patóxeno (Capítulo III).

Os obxectivos específicos distribuíronse en tres capítulos como segue:

1. Desenvolvemento e optimización dun método de PCR a tempo real para a detección e cuantificación específica do probiótico *Phaeobacter* 27-4 e dos patóxenos de rodaballo *L. anguillarum* 90-11-287 e *V. splendidus* DMC-1 nos organismos involucrados no cultivo larvario do rodaballo: microalgas (*Isochrysis galbana*), empregadas no enriquecemento en ácidos graxos das presas vivas, rotíferos (*Brachionus plicatilis*) e *Artemia* empregados como presas vivas, e larvas de rodaballo (*Psetta maxima*) (Capítulo I).

2. Estudo da colonización e o tempo de residencia dos patóxenos *Listonella anguillarum* 90-11-287 e *Vibrio splendidus* DMC-1 no rotíferos *Brachionus plicatilis* (Capítulo II), incluíndo:

    2.1. A avaliación da colonización e o seguimento do tempo de residencia de ámbolos dous patóxenos nos rotíferos trala súa transferencia ós tanques de cultivo larvario e mantidos nas condicións habituais do cultivo larvario do rodaballo, mediante a aplicación da PCR a tempo real.

    2.2 A análise dos cambios nas poboacións bacterianas dos rotíferos e auga, como consecuencia da introdución dos patóxenos mediante o uso dunha técnica de DGGE.

3. Estudo da aplicación de *Phaeobacter* 27-4 en biopelículas, incluíndo:

    3.1. A selección dun soporte e das condicións de cultivo de *Phaeobacter* 27-4, baseándose:

        3.1.1. No crecemento e na formación de biopelículas de *Phaeobacter* 27-4 sobre diferentes soportes usados en acuicultura (bolas plásticas,

bolas de vidro expandido e cilindros cerámicos) e diferentes condicións de cultivo (empapados e somerxidos con ou sen axitación)

3.1.2. Na actividade antagonista dos biofiltros de *Phaeobacter* 27-4 fronte *L. anguillarum* 90-11-287 e *V. splendidus* DMC-1 en experimentos de inactivación ou inhibición de crecemento levados a cabo en microcosmos.

3.1.3. No tempo de residencia da bacteria probiótica nos biofiltros preparados con diferentes soportes e mantidos nas condicións de cultivo larvario do rodaballo.

3.2. Unha vez seleccionado un soporte e as condicións de cultivo de *Phaeobacter* 27-4, demóstrase:

3.2.1. O efecto dos biofiltros de *Phaeobacter* 27-4 fronte a *L. anguillarum* 90-11-287 en auga verde baixo as condicións de cultivo larvario e diferentes niveles de nutrientes.

3.2.2. O efecto probiótico *in vivo* dos biofiltros de *Phaeobacter* 27-4 sobre larvas de rodaballo infectados con *L. anguillarum* 90-11-287 introducido vía rotíferos.

3.2.3. A relación entre a supervivencia larvaria do rodaballo e a presenza de *Phaeobacter* 27-4 ou a modificación da microbiota bacteriana das larvas e a auga.

8.3. Capítulo I: Detección e cuantificación mediante PCR a tempo real do probiótico *Phaeobacter* 27-4 e dúas vibrionaceas patóxenas de peixes en microalgas, rotíferos, *Artemia* e larvas de rodaballo (*Psetta maxima*) no comezo da alimentación esóxena

O seguimento das cepas introducidas nos sistemas de cultivo larvario é esencial para o desenvolvemento de protocolos e estratexias para a aplicación industrial dos

probióticos. As colonias de *Phaeobacter* 27-4 pódense distinguir e cuantificar facilmente sobre placas de Agar Mariño debido á produción dun pigmento castaño-amarelado (Hjelm *et al.*, 2004a, b). Non obstante, como en tódolos métodos baseados no cultivo, precísase tempo para o crecemento das colonias dificultando o seguimento diario de, por exemplo, os niveis do probiótico no sistema. Así mesmo, para a determinación do efecto *in vivo* dos probióticos, incluíndo lugares de acción, tamén se require un seguimento continuo das bacterias potencialmente patóxenas. A cuantificación das *Vibrionaceae* patóxenas mediante os métodos convencionais de cultivo en placa é complicada, véndose ademais a súa cantidade infravalorada cando se estima sobre un medio selectivo como o Tiosulfato-Citrato-Bile-Sacarosa (TCBS) (Mizuki *et al.*, 2006).

Así, neste capítulo desenvolveuse e optimizouse unha ferramenta de PCR a tempo real para seguir e cuantificar especificamente ó probiótico *Phaeobacter* 27-4 así como ós patóxenos, *Listonella* (*Vibrio*) *anguillarum* 90-11-287 e *Vibrio splendidus* DMC-1 dentro dos sistemas de cultivo larvario.

Phaeobacter 27-4, *L. anguillarum* 90-11-287 e *V. splendidus* DMC-1 cuantificáronse en cultivos puros e en mesturas con microalgas (*Isochrysis galbana*), rotíferos (*Brachionus plicatilis*), nauplios de *Artemia* ou larvas de rodaballo (*Psetta máxima*) mediante PCR a tempo real baseada en cebadores dirixidos a loci xenéticos que codifican para a función de probiosis ou virulencia. O protocolo optimizado empregouse no estudo da bioencapsulación e mantemento do probiótico e dos patóxenos nos rotíferos e para a detección e cuantificación de *Phaeobacter* e *L. anguillarum* en larvas de rodaballo alimentadas con rotíferos infectados coas diferentes bacterias nun experimento levado a cabo en planta piloto.

O protocolo de PCR a tempo real desenvolvido demostrou ser reproducible e específico, requirindo rectas de calibrado diferentes en función do organismo presente e podendo usarse para a detección e cuantificación de probióticos do xénero *Phaeobacter* e patóxenos dos xéneros *Listonella* e *Vibrio*, bioencapsulados en rotíferos e en larvas de rodaballo.

A importancia do estudo realizado reside en que este método permite o seguimento e a cuantificación de bacterias probióticas e patóxenas do rodaballo en experimentos *in vivo*, facéndoa unha ferramenta moi útil para a detección destas bacterias nas unidades industriais de cultivo larvario.

8.4. Capítulo II: Diferente colonización e tempo de residencia dos patóxenos de peixes *Listonella anguillarum* e *Vibrio splendidus* no rotífero *Brachionus plicatilis* determinados mediante PCR a tempo real e DGGE

Listonella anguillarum 90-11-287 e *Vibrio splendidus* DMC-1 foron incorporados no rotífero *Brachionus plicatilis* os que se mantiveron nas condicións de cultivo larvario para determinar a colonización e o tempo de residencia de ámbolos dous patóxenos nos rotíferos.

A PCR a tempo real aplicouse para detectar e cuantificar especificamente ós dous patóxenos. *L. anguillarum* colonizou ós rotíferos máis ca *V. splendidus* e ámbolos dous pasaron dos rotíferos á auga, tras a transferencia dos rotíferos ós tanques de cultivo larvario. *V. splendidus* medrou e pasou a ser predominante na auga dos tanques. Os dous patóxenos permaneceron no rotífero a na auga o tempo suficiente para a infección das larvas de peixes, pero o diferente comportamento observado para estas dúas cepas podería determinar diferentes patróns de infección, preferiblemente por inxestión das presas ou por contacto coa auga circundante.

O efecto de *L. anguillarum* e *V. splendidus* na comunidade bacteriana dos rotíferos e da auga nos tanques de cultivo analizouse mediante DGGE dos fragmentos amplificados do xene ARN 16S. A comunidade bacteriana dos rotíferos non presentou unha marcada dominancia de especies. A incorporación de *L. anguillarum* ou *V. splendidus* non diminuíu a diversidade bacteriana nos rotíferos e os cambios observados na microbiota bacteriana poderían ser explicados mediante o intercambio bacteriano entre os rotíferos e a auga. Os principais grupos bacterianos identificáronse mediante secuenciación do ADN extraído dos illados en Agar Mariño (AM) e das bandas recortadas dos xeles de DGGE. Só dúas bandas da DGGE correspondéronse con bacterias illadas en AM, o que suxire que os grupos bacterianos presentes nos rotíferos pode que non sexan facilmente cultivables. A comunidade bacteriana dos rotíferos estaba composta por bacterias Gram negativas pertencentes ós grupos α-Proteobacteria (*Ruegeria* spp), γ-Proteobacteria (*Alteromonas alvinellae*, *Marinobacter* sp, unha bacteria da familia *Oceanospirillaceae* e *Pseudoalteromonas* sp), *Cytophaga-Flexibacter-Bacteroides* (*Polaribacter dokdonensis*, *Roseivirga spongicola* e *Tenacibaculum soleae*) e unha bacteria Gram positiva (*Microbacterium* sp).

8.5. Capítulo III: Aplicación de biofiltros coa cepa probiótica *Phaeobacter* 27-4 fronte a infeccións bacterianas da familia *Vibrionaceae* no cultivo larvario do rodaballo (*Psetta maxima*)

O crecemento e o antagonismo (*in vivo* e *in vitro*) da cepa probiótica *Phaeobacter* 27-4 estudouse con diferentes soportes empregados en biofiltros na acuicultura (bolas de plástico, bolas de vidro expandido e cilindros cerámicos) e en diferentes condicións de cultivo (enchoupado e somerxido con ou sen axitación). A adhesión e o crecemento de *Phaoebacter* ademais da formación de microcolonias con forma de roseta e a consecuente formación dunha biopelícula foi diferente dependendo do soporte e das condicións de cultivo. Así, só nos cilindros cerámicos incubados somerxidos sen axitación, se observou unha biopelícula formada por varias capas con microcolonias con forma de roseta.

A actividade antagonista *in vitro* dos biofiltros de *Phaeobacter* 27-4 fronte a *L. anguillarum* 90-11-287 e *V. splendidus* DMC-1 foi diferente cando o probiótico se inmobilizou en diferentes materiais. O grado de antagonismo viuse afectado polo soporte e polas condicións de cultivo do probiótico. En ausencia de nutrientes, a inactivación foi semellante para ámbolos dous patóxenos, pero a inhibición do crecemento causada polo probiótico en presenza de nutrientes foi meirande para *L. anguillarum* que para *V. splendidus*. En presenza de *Phaeobacter*, *V. splendidus* medrou máis que *L. anguillarum* e promoveu un crecemento menor do probiótico no medio de cultivo. A presenza de *Phaeobacter* diminuíu a adhesión dos dous patóxenos ós soportes cerámicos e de vidro expandido pero non ós de plástico.

A permanencia de *Phaeobacter* 27-4 nos biofiltros estudouse en tanques con auga verde mantidos nas condicións de cultivo larvario. *Phaeobacter* amosou unha mellor permanencia nos soportes porosos, comparados cos de plástico, mostrando unha menor pendente de desprendemento dos cilindros cerámicos. Nos perfiles de DGGE, os biofiltros cerámicos amosaron o menor número de bandas, o que indica que a presenza de *Phaeobacter* nestes biofiltros evita a colonización por outras bacterias.

Os cilindros cerámicos seleccionáronse para a preparación dos biofiltros que se empregaron nos ensaios en planta piloto debido á capacidade de *Phaeobacter* 27-4 en ese soporte para: *i*) desenvolver unha biopelícula formada por rosetas, *ii*) antagonizar dous patóxenos (*L. anguillarum* e *V. splendidus*), e *iii*) permanecer por máis tempo nos

biofiltros cando se manteñen nas condicións de cultivo larvario. Adicionalmente, a cerámica é un material resistente, sendo isto unha vantaxe para o escalado ás plantas de acuicultura.

En experimentos a pequena escala levados a cabo nas condicións de cultivo larvario e diferentes niveles de nutrientes, os biofiltros cerámicos maduros de *Phaeobacter* 27-4 reduciron a concentración de *L. anguillarum* 90-11-287 e *Vibrionaceae* totais na auga dos tanques con diferentes niveles de nutrientes.

Os biofiltros de *Phaeobacter* 27-4 reduciron significativamente a mortalidade acumulada en larvas de rodaballo infectadas co patóxeno *L. anguillarum* 90-11-287 mediante a diminución dos niveles do patóxeno e da concentración das *Vibrionaceae* totais, principalmente na auga. Ademais, o biofiltro probiótico reduciu a turbidez nos tanques de cultivo.

A análise da DGGE amosou que a incorporación de *L. anguillarum* ou dos biofiltros maduros de *Phaeobacter* non desprazaron ou modificaron significativamente a microbiota bacteriana presente nas larvas, a que ten unha diversidade bacteriana baixa. Non obstante, a presenza dos biofiltros probióticos diminuíu a carga bacteriana na auga dos tanques. Os cambios observados na comunidade bacteriana das larvas de rodaballo reflectiron a influenza dos rotíferos empregados como alimento, namentres que na comunidade bacteriana da auga os cambios víronse marcados pola desaparición de bandas en presenza dos biofiltros probióticos.

Os biofiltros probióticos actuaron como un reservorio das bacterias probióticas no sistema de cultivo larvario, mantendo o nivel do probiótico na auga dos tanques a lo menos dez días, período no que as larvas se alimentan con rotíferos e que é considerada crítica para a supervivencia larvaria. Unha vantaxe importante dos biofiltros é que o seu uso non precisa de adicións repetidas do probiótico, evitando cultivos a longo prazo e a bioencapsulación nos rotíferos. Este aspecto simplifica a transferencia potencial de esta metodoloxía ás plantas industriais de cultivo larvario.

8.6. Bibliografía

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