Universidade de Aveiro Departamento de Biologia

Ano 2013

Lília Cristiana da Silva Mateus

Terapia fágica em aquacultura: uso de cocktails de fagos e de lisozima

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Terapia fágica em aquacultura: uso de cocktails de fagos e de lisozima

Phage therapy in aquaculture: use of phage cocktails and lysozyme

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Microbiologia, realizada sob a orientação científica da Doutora Maria Adelaide de Pinho Almeida, Professora Auxiliar do Departamento de Biologia da Universidade de Aveiro, e coorientação da Doutora Liliana Andreia dos Santos Costa, bolseira de investigação do Departamento de Biologia da Universidade de Aveiro.

This work was supported by funding FEDER through COMPETE - Programa Operacional Factores de Competitividade, and by National funding through FCT-Fundação para a Ciência e Tecnologia (Foundation for Science and Technology), within the research project FCOMP-01-0124-FEDER-013934 and also to Centre for Environmental and Marine Studies, University of Aveiro (CESAM, project Pest-C/MAR/LA0017/2011).

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agradecimentos

Quero expressar, em especial, os meus sinceros agradecimentos à minha orientadora de dissertação, Doutora
Adelaide Almeida, pela total disponibilidade, pelo apoio incansável, críticas construtivas, dedicação, amizade, paciência e ensinamentos transmitidos, que contribuíram para o sucesso desta etapa.

Agradeço igualmente à minha coorientadora, Doutora Liliana Costa, pelo sentido crítico, pela amizade, conhecimentos transmitidos, pela disponibilidade e paciência, sem ela a realização desta etapa também não teria sido possível.

Agradeço à Yolanda, aluna de Doutoramento, pela amizade, pela sua preciosa colaboração, pela disponibilidade, pela motivação e por todo o apoio dado durante este percurso.

À Carla e à Inês, alunas de Doutoramento, pelos conhecimentos transmitidos e pela pronta disponibilidade.

À Marina, à Carina e à Mariana, colegas de laboratório, pelo companheirismo e por todos os bons momentos passados.

Não posso deixar de agradecer às minhas amigas, Janete, Tânia Ribeiro e Tânia Martins o apoio incondicional. Obrigada por todas as palavras de incentivo sempre que precisei, e pela inesgotável amizade.

Ao meu irmão Ricardo, à Ana e ao meu afilhado Gonçalo, obrigada por me apoiarem em todos os momentos.

Por último, não menos importante, agradeço aos meus pais pelo amor, pela compreensão e pela paciência durante esta caminhada, sem o vosso apoio não teria sido possível a realização desta etapa. Obrigada!

palavras-chave

Terapia fágica, bacteriófagos, bactérias patogénicas de peixes, lisozima, cocktail, aquacultura.

Resumo

A aquacultura é um dos setores da área alimentar que mais tem crescido nos últimos anos. No entanto, o aparecimento de microrganismos patogénicos, incluindo bactérias multirresistentes, e a sua disseminação no meio ambiente tornou-se um problema para a indústria de aquacultura. Este facto leva a que seja necessário o desenvolvimento de estratégias menos lesivas para o ambiente de forma a permitir o crescimento sustentável da aquacultura. A terapia fágica surge como uma alternativa potencialmente viável e eficaz para inativar bactérias patogénicas em aquacultura. O principal objetivo deste trabalho consistiu na avaliação da eficácia da terapia fágica para inativar bactérias patogénicas de peixes. Foi avaliado o efeito do uso de cocktails de fagos e de lisozima na eficiência da terapia fágica. Os ensaios de terapia fágica foram realizados com a bactéria *Vibrio parahaemolyticus* e com três fagos produzidos sobre esta bactéria (VP-1, VP-2 e VP-3). A dinâmica de interação fago-bactéria foi caracterizada em meio de cultura Tryptic Soy Broth através da quantificação do hospedeiro por incorporação e da quantificação dos fagos pela técnica da dupla camada de agar. Os três fagos foram testados isoladamente e em cocktails de dois ou três fagos. A eficiência de inactivação bacteriana pelos três fagos foi testada com diferentes concentrações de lisozima (gama 0,8 μg mL^{-1} a 20 mg mL^{-1}). Como, para aplicar com sucesso a terapia fágica, é importante ter informação sobre as características dos fagos, a gama de hospedeiros, a sua sobrevivência na água da aquacultura, bem como o seu número e tempo de explosão, foram determinados. Para o estudo da gama de hospedeiros de bacteriófagos usou-se a infeção cruzada. Para determinar a sobrevivência dos fagos na água marinha foi usada a técnica da dupla camada de agar. O número e o tempo de explosão foram determinados através da determinação das curvas de crescimento síncrono. O uso de cocktails de dois e três fagos foi significativamente mais eficaz (redução de 4 log após 2 h de tratamento) do que o uso dos fagos VP-1, VP-2 e VP-3 sozinhos (redução 0,6, 0,8, e 2,6 log para os fagos VP-1, VP-2 e VP-3, respectivamente, após 2 h de tratamento). A combinação de lisozima e fago apresentou melhor atividade inibidora em comparação com a atividade do fago sozinho. Os fagos VP-1 e VP-2 foram mais eficazes na inativação bacteriana (redução de cerca 4 log após 6 - 8 h de tratamento) na presença de altas concentrações de lisozima do que o fago VP-3. No entanto, o fago VP-3 foi mais eficaz na presença de baixas concentrações de lisozima (redução de 3,2 log, depois de 2 h de tratamento). Os resultados da infeção cruzada mostraram que os fagos de *Vibrio parahaemolyticus* inoculados noutras bactérias infetaram também *Vibrio anguillarum* e *Aeromonas salmonicida*, apresentando uma eficiência de infeção elevada. Os ensaios de sobrevivência dos fagos na água de aquacultura mostraram que estes permanecem viáveis por longos períodos de tempo (mais de 5 - 7 meses). O fago VP-3 apresentou um número de explosão maior e um período latente menor (42 e 40 min, respetivamente), do que os outros dois fagos (9 e 120 min e 15 e 90 min, respetivamente, para o fago VP-1 e VP-2). Em conclusão, a utilização de cocktails de fagos parece ser uma abordagem eficaz para o tratamento de vibrioses. A inativação bacteriana é mais eficiente e ocorre mais cedo quando são usados os cocktails de fagos, mas a sua utilização *in vitro* não impede o recrescimento bacteriano após o tratamento, retardando, no entanto, o recrescimento da bactéria. A aplicação de fagos com lisozima, para eliminar ou reduzir bactérias patogénicas de peixes em aquacultura, pode ser uma estratégia promissora, nomeadamente quando os fagos disponíveis são menos eficientes. A utilização de fagos com um alto número de explosão e um período latente curto aumenta claramente a eficiência da terapia fágica.

Keywords Phage therapy, bacteriophages, fish pathogenic bacteria, lysozyme, cocktail, aquaculture.

Abstract

Aquaculture is one of the fastest growing food industry sectors in the world in recent years. However, the appearance of pathogenic microorganisms, including multirresistant bacteria, and their dissemination in the environment has become a problem for the aquaculture industry. This means that it is necessary to develop less harmful strategies to the environment to allow a sustainable growth of the aquaculture systems. Phage therapy emerges as a potential alternative to inactivate pathogenic bacteria in aquaculture. The main objective of this study was to assess the efficacy of phage therapy to inactivate fish pathogenic bacteria. The use of phage cocktails and lysozyme was also evaluated on the efficiency of phage therapy. The phage therapy assays were performed with the bacterium *Vibrio parahaemolyticus* and with three phages produced on this bacterium (VP-1, VP-2 and VP-3). The dynamics of phage-bacteria interaction was characterized in Tryptic Soy Broth through host and phage quantification, respectively by pour plate and by the double-layer agar technique. The three phages were tested alone and in cocktails of two or three phages. The efficiency of the bacterial inactivation by the phages was tested at different lysozyme concentrations (range 0.8 $\mu g\ mL^{-1}$ to 20 $mg\ mL^{-1}$). As the selection of bacteriophages is a key factor for the success of phage therapy, the host range, their survival in aquaculture water, as well as the burst size and the explosion time, were determined. The cross-infection was used to determine the phage host range. To determine the survival of the phages in marine water, the double-layer agar technique was used. The burst size and the explosion time were calculated by the one-step growth curve analysis. The use of cocktails of two and three phages was significantly more effective (reduction of 4 log at 2 h of treatment) than the use of the VP-1, VP-2 and VP-3 phages alone (reductions of about 0.6, 0.8 and 2.6 log, at 2 h of treatment respectively for the VP-1, VP-2, and VP-3 phages). The combination of phage plus lysozyme showed a better inhibitory activity when compared with the activity of the phage alone. The VP-1 and VP-2 phages were more efficient to inactivate the *Vibrio* (reduction of about 4 log after 6 - 8 h treatment), in the presence of high concentrations of lysozyme, than the VP-3 phage. However, the VP-3 phage was more efficient in the presence of low concentrations of lysozyme (reduction of 3.2 log after 2 h of incubation). The results of the cross-infection showed that the phages of *Vibrio parahaemolyticus* also infect *Vibrio anguillarum* and *Aeromonas salmonicida* with high efficiency. The assays of phage survival in aquaculture water showed that the phages remain viable for long time periods (more than 5 - 7 months). The VP-3 phage presented a higher burst size and a shorter latent period (42 and 40 min, respectively) than the other two phages (9 and 15 and 120 min and 90 min, respectively, for the VP-1 and VP-2 phages). In conclusion, the use of phage cocktails appears to be an effective approach to treat vibriosis. Bacterial inactivation is more efficient and occurs earlier when the phage cocktails are used, but their use *in vitro* does not prevent bacterial regrowth after treatment. However, the use of phage cocktails retarded the regrowth of the bacteria. The application of phages with lysozyme to eliminate or reduce fish pathogenic bacteria in aquaculture can be a promising strategy, namely when less effective phages are available. Besides, the use of phages with a high burst size and a short latent period clearly increase the efficiency of phage therapy**.**

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Acronyms and Abbreviations

ANOVA	Analysis of variance
CFU	Colony forming units
CFU mL^{-1}	Colony forming units per milliliter
CWBD	C-terminal cell wall binding domain
ds	Double-stranded
HACCP	Hazard Analysis and Critical Control Points
ICTV	International Committee for taxonomy
LPS	Lipopolysaccharide
MOI	Multiplicity of infection
PFU	Plaque forming units
PFU mL^{-1}	Plaque forming units per milliliter
PG	Peptidoglycan
SAR	Signal arrest release
ss	Single-stranded
VNN	Viral nervous necrosis
TSA	Tryptone Soy Agar
TSB	Tryptic Soy Broth
WHO	World Health Organization

Chapter 1: Introduction

1.1 Bacteriophages

1.1.1 Discovery of bacteriophages

Bacteriophages, or simply phages, are viruses that have the ability to infect bacteria, and, in the case of lytic phages, cause lysis of the bacteria leading to their subsequent death (Sulakvelidze, *et al*., 2001). These form the largest group of all viruses, and have the ability to colonize every habitat conceivable in nature and can be found in large quantities. The group where the bacteriophages are found is quite diverse, being indisputably the oldest one (Ackermann, 2001, Ackermann, 2007). They exist in high concentrations in natural environments, it is estimated that the presence of bacteriophages in the world is 10^{31}, which is equivalent to about 109 tones, making them the most abundant viruses the on earth (Kropinski, 2006, Hanlon, 2007).

The discovery of these viruses is involved in a great controversy because, in 1896, Ernest Hanking, a british bacteriologist, noted the presence of an unidentified substance in the waters of an indian river. That substance limited the spread of cholera epidemics. Two years later, a russian bacteriologist, Nikolay Gamaleya, observed a similar phenomenon while working with *Bacillus subtilis*. About twenty years after the observations of Ernest Hanking, a medically trained english bacteriologist named Frederick Twort, reported something similar and put forward the possibility that the observed results could be due to a virus. For various reasons, such as financial ones, the bacteriologist Twort failed to pursue his investigations (Kropinski, 2006, Hanlon, 2007). In 1917, Felix d'Herelle, a franco-canadian microbiologist, officially discovered the bacteriophages. During his studies, d'Herelle filtered fecal samples of several patients, mixed and incubated it with *Shigella* strains isolated from the patients. A portion of this mixture was placed in experimental animals, due to the fact that part of his study of focused on the development of a vaccine against bacterial dysentery. The other part of the mixture was plated in agar medium in order to observe bacterial growth. After incubation, d'Herelle noted the appearance of white spots, that he gave the name of plates. After his observation he was convinced that the invisible microbes responsible for that plates were viruses and that they

were able to multiply indefinitely causing the lysis of bacteria that are necessary for their survival (Sulakvelidze, *et al*., 2001). After the discovery of bacteriophages, d`Herelle introduced the term phage therapy, that was regarded as a possible method of treatment against bacterial infectious diseases (Karen, 2001, Sulakvelidze, *et al.*, 2001). This method of treatment was used to treat and prevent bacterial infection diseases in the former Soviet Union and Eastern Europe, however, was abandoned by the West in the 1940 with the appearance of the antibiotics (Chanishvili, *et al.*, 2001, Matsuzaki, *et al.*, 2005). The emergence of pathogenic bacteria resistant to antibiotics, including multirresistant bacteria, has recently motivated the western scientific community to revaluate phage therapy as a valid option for the treatment of bacterial infections (Krylov, 2001, Gill & Hyman, 2010). Currently, beyond phage therapy of humans pathogenic bacteria, phage therapy is also being studied as a treatment for animals and plant diseases caused by bacteria, and is already used as a means of controlling both human pathogenic and spoilage bacteria in foods (Balogh, *et al.*, 2010, Gill & Hyman, 2010, Kutter, *et al.*, 2010).

1.1.2 Bacteriophages morphology

In morphological terms, the bacteriophages may have different sizes and shapes, but mostly of them exhibit a capsid, collar and tail. (Figure 1.1) (Hanlon, 2007). The head (or capsid) is a protein shell often in the shape of an icosahedron and usually comprises double-strand (ds) DNA. The capsid is organized into capsomeres, whose main function is to protect the genetic material (Ackermann, 2001, Goodridge & Abedon, 2003, Hanlon, 2007). The capsid has three important functions during the phage life cycle: (1) protect the phage genome during its extracellular phase, (2) enable the adsorption of the phage, fixing the virus to the host bacterium (in *Caudovirales*), and (3) the subsequent delivery of the phage genome into the host cytoplasm (Goodridge & Abedon, 2003). The phage genome can vary between 17 kb and 500 kb. However, despite the size of the phage genome, it doesn’t have the necessary machinery for the production of energy and doesn’t also have ribosomes for protein synthesis (Goodridge & Abedon, 2003). Regarding the tail, six fibers are usually linked to the tail, containing receptors on their ends that recognize binding sites on the surface of the bacterial cell. However, not all phages have tails and tail fibers and in this situation there are other attachment mechanisms (Goodridge & Abedon, 2003, Hanlon,

2007). Phages without a tail, like the MS2 phage, only infect bacterial cells that contain a certain type of plasmid, called the conjugative plasmid, which allows the bacterial cell to function as a donor in conjugation. This is because these viruses infect bacteria by first attaching to the pili (Madigan, 2011).

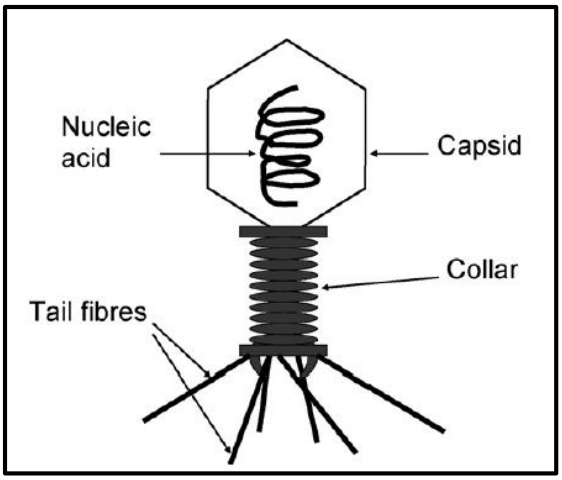

Figure 1.1 - Schematic representation of a typical bacteriophage (adapted from Hanlon, 2007).

1.1.3 Taxonomy of bacteriophages

The International Committee for Taxonomy of Viruses (ICTV) is presently responsible for the classification of the viruses (Ackermann, 2003).

The taxonomic classification of bacteriophages takes into account their size and shape as well as their type of nucleic acid (Table 1.1 and Figure 1.2). The dsDNA phages with tail are classified in the *Caudovirales* order. These bacteriophages belong to the *Caudovirales* order, based on the bacteriophage tail structure, are divided into three families: (1) *Moyviridae* , in which viruses have a contractile tail constituted by a hem and a central tube, (2) *Siphoviridae*, in which viruses have long not contractible tails, and (3) *Podoviridae*, in which viruses have no contractile and short tails (Ackermann, 2003, Ackermann, 2007, Drulis-Kawa, *et al.*, 2012). The other non-tailed phages, are classified into ten families (Table 1.1), and are cubic, filamentous, or pleomorphic and contain double-stranded or single stranded DNA or RNA as the genome (Ackermann, 2007).

Although it was assumed that most of the viruses in seawater contain DNA and infect bacteria, presently it is known that the abundance of RNA containing viruses in the

ocean, which almost exclusively infect eukaryotes, rivaled or even exceeded that of DNA viruses in samples of coastal seawater. It has been recently discovered new single-stranded RNA (ssRNA) viruses, that infect marine protists, which are classified as members of the order *Picornavirales*. A new genus in the family *Reoviridae* has also been reported as a double-stranded RNA virus that infect the abundant marine prymnesiophyte *Micromonas pusilla* (Steward, *et al.*, 2013).

Table 1.1 Basic properties of phage families (adapted from Ackermann, 2003).

Shape	Family	Nucleic acid	Characteristics	Example
Tailed	*Myoviridae*	DNA, ds, L	Contractile tail	T4
	Siphoviridae	DNA, ds, L	Long, noncontractile tail	λ
	Podoviridae	DNA, ds, L	Short tail	T7
Polyhedral	*Microviridae*	DNA, ss, C		φX174
	Corticoviridae	ds, C, T	Complex capsid, lipids	PM2
	Tectiviridae	ds,L	Internal lipoprotein vesicle	PRD1
	Leviviridae	RNA, ss, L		MS2
	Cystoviridae	ds, L, S	Envelope, lipids	φ6
Filamentous	*Inoviridae*	DNA, ss, C	Filaments or rods	Fd
	Lipothrixviridae	ds, L	Envelope, lipids	TTV1
	Rudiviridae	ds, L	Resembles TMV	SIRV1
Pleomorphic	*Plasmaviridae*	DNA, ds, C, T	Envelope, lipids, no capsid	L2
	Fuselloviridae	ds, C, T	Spindle-shaped, no capsid	SSV1

Legend: C, circular; L, linear; S, segmented; T, superhelical; 1, single-stranded; 2, double-stranded.

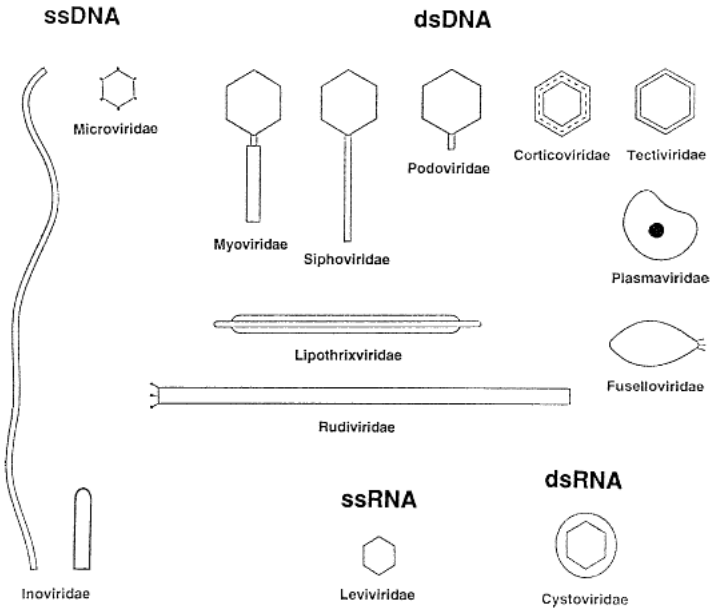

Figure 1.2 - Schematic representation of the morphology of bacteriophages (adapted from Ackermann, 2003).

1.2.4 The phage life cycle

There are two important ways in which viruses interact with their hosts: the infection cycle with a lytic pathway and the lysogenic infection cycle (Lenski, 1988, Hanlon, 2007). In the lytic cycle, as shown in Figure 1.3 the phage injects its genome into the host cell (Hogg, 2005). To multiply itself, the phage needs to cause lysis of the host cell to release the newly formed phages (Skurnik & Strauch, 2006). The viruses find their host by the presence of cell surface components, or, in some cases, binding sites may be present in the cell capsule, flagella, or even pili, allowing the recognition and following attachment (Fischetti, 2005, Hanlon, 2007). At the beginning, this connection is made randomly and is reversible, but later becomes irreversible and is followed by the transfer of the phage genetic material to the host cell. The injection of the phage genome into a bacterial cell can occur by a variety of mechanisms (depending on the morphology of the viruses), but often involves the contraction of the tail and formation of a hole inside the bacterial cell wall. The bases of the phage DNA are often modified to protect against the attack by restriction enzymes and cellular nucleases. The viral genome is transcribed by RNA polymerases of the host cell, producing premature mRNA. The premature mRNA function is to take over

the metabolic machinery of the bacterium and redirect the metabolic processes to the manufacture of new viral components (Hanlon, 2007).

After the replication and assembly of new phage particles within the host cell, the new phages are released to the environment (Hanlon, 2007). Almost all of the dsDNA phages develop enzymes that attack the bacterial peptidoglycan, like lysozymes that act at the sugar links, endopeptidases that break peptide bonds or amidases that act on amide links (Fischetti, 2005). These lytic enzymes (usually called muralytic enzymes or endolysins), coded by the phage genome, are produced within the cytoplasm but require another enzyme to allow them to cross the cytoplasmic membrane to reach its substrate. This enzyme is a holin that ruptures the membrane, allowing the lysin to degrade the peptidoglycan (Young, *et al.*, 2000, Fischetti, 2005). The holin controls the timing of the cell lysis and the release of the phage progeny. The filamentous phage can escape the host cell by extrusion through the cell wall without causing the destruction of the host, these phages did not present relevance for phage therapy (Hanlon, 2007). The period of time between the attachment of a phage particle to the cell surface and the release of the newly synthesized phages is called the latent period, sometimes also known as the burst time (Hogg, 2005).

Temperate phages are viruses that don't enter automatically on a lytic cycle, the phage genome will integrate the genome of the host cell (Figure 1.3). By being included in the host genome, the genetic material of the phage will be replicated together with the genome of the bacteria, remaining in a dormant state as a prophage for long periods of time (Figure 1.3) (Hogg, 2005, Skurnik & Strauch, 2006). The temperate phages induce a state of lysogeny in the bacterial host (Hanlon, 2007).

Cells can undergo multiple rounds of division but, occasionally, one will spontaneously lyse and release progeny phage. Alternatively, a population of lysogenic cells can be induced to lyses by submitting them to stress and treatment with mutagens or by exposure to ultraviolet light. A few temperate phages, such as Mu, can switch between lysogeny and lytic growth under the influence of elevated temperatures in the host stationary phase (Ranquet, *et al.*, 2005). The prophage directs the synthesis of a repressor protein which blocks the transcription of its own genes and also those of closely related bacteriophages. The presence of a prophage may therefore confer a certain type of bacterial cell immunity to other infections by phages. Lysogenic bacteria may have other

advantages, in terms of acquisition of genes that confer increased virulence or pathogenicity. When one prophage escapes regulation by the repressor, its DNA is cut free inducing a lytic cycle. But, excision of the prophage DNA is often imprecise and bacterial genes adjacent to the prophage DNA may be incorporated into infectious phage DNA and then transferred into host cells. This process is called transduction, and is responsible for the horizontal transfer of genes from one bacterial cell to another. Examples of virulence genes include those used for host attachment, invasion and survival, as well as for the production of toxins. Temperate phages are not suitable candidates for phage therapy since they can't immediately destroy the host bacteria (Brüssow, *et al.*, 2004, Hanlon, 2007).

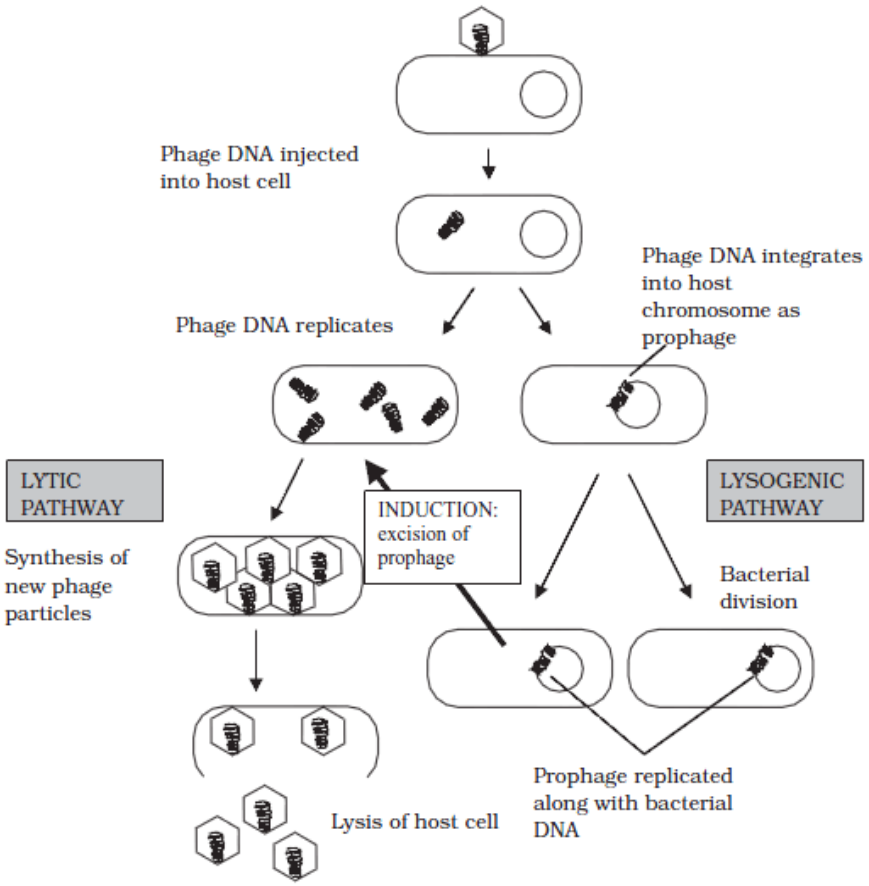

Figure 1.3 - Bacteriophages lytic and lysogenic cycles (adapted from Hogg, 2005).

1.2.5 Effect of lysins on phage activity

The phages can use two forms to release the new viral particles from the bacterial hosts. In filamentous phages, the progeny is unceasingly extruded from the bacterial cells without harming the bacteria, through the use of phage encoded specific enzymes that interfere with host enzymes that are responsible for peptidoglycan synthesis (García, *et al.*, 2010), while non-filamentous phages breakdown the host cell wall by phage encoded lytic enzymes (Hermoso, *et al.*, 2007, García, *et al.*, 2010).

The lytic enzymes (also called endolysins or lysins) used to destroy the bacterial cell wall are enzymes that are encoded by the phage genome, and are produced during the late phase in the lytic cycle to degrade the bacterial cell wall, and act on the cell wall from inside the cell facilitating the release of the virions (Hogg, 2005, Hermoso, *et al.*, 2007, Drulis-Kawa, *et al.*, 2012). The lytic enzymes used to infect the cell are components of the virion tail that are able to locally digest the cell wall from the outside to facilitate the injection of the phage genome into the host cell (Hogg, 2005, Hermoso, *et al.*, 2007). This kind of lytic enzymes are widespread in the virions of phages infecting Gram-positive or Gram-negative bacteria (Hermoso, *et al.*, 2007). A classic example is the T4 phage lysozyme that is inserted into a baseplate protein of the tail tube, at the tip of the tube (Hermoso, *et al.*, 2007).

Lysins can be classified according to their catalytic activity as lysozymes or muramidases, glucosaminidases, N-acetylmuramoyl-L-alanine-amidases (NAM-amidases), endopeptidases and lytic transglycosylases. Glucosaminidases, lysozymes and lytic transglycosylases act on the sugar moiety (glycosidases), whereas endopeptidases cleave the peptide cross-bridge and NAM-amidases hydrolyze the amide bond connecting the sugar and peptide constituents of peptidoglycan (Moak & Molineux, 2004, Hermoso, *et al.*, 2007, García, *et al.*, 2010). These lytic enzymes, namely lysozymes, are also produced by other eukaryotic and prokaryotic organisms, being involved in non-specific defence mechanisms (Burge, *et al.*, 2007). Muramidases and amidases that hydrolyze the most conserved bonds in the peptidoglycan appear to be the most widely spread (Fischetti, 2008).

Normally, lysins have no signal peptide, accessing to the peptidoglycan of the cell through the use of small hydrophobic protein, denominated holin, allowing the lysins to

cross the cytoplasmic membrane and gain access to the cell wall (Loessner, 2005, Borysowski, *et al.*, 2006, Hermoso, *et al.*, 2007). However, a few number of lytic enzymes contain signal peptides that are recognized by the host general secretion pathway (García, *et al.*, 2010). In this case, a typical host sec system is used to reach the peptydoglican. Some lysins that are exported by the host sec system comprehend N-terminal signal sequences that function as a type II signal anchor or uncleaved signal peptide. This signal sequence has been nominated signal arrest release (SAR) sequence because of the ability of the protein to escape from the membrane and posterior release into the periplasm (Hermoso, *et al.*, 2007).

Phage lysins can also be classified according to their structure as globular or modular composed. The globular structure is built of a single catalytic domain and the modular structure is composed by two domains: N-terminal catalytic domain (CD), and C-terminal cell wall binding domain (CWBD), or three domains (with an additional mid protein domain with enzymatic activities). Normally, lysins from Gram-negative specific phages are characterized by a globular structure, single module structure, whereas an additional substrate-binding domain is typical of Gram-positive specific phages. Depending on the type of the targeted bond in the peptidoglycan CD cab, they exhibit different enzymatic activities. The C-terminal cell wall binding domain (CWBD) confers some degree of specificity to the enzymes (Drulis-Kawa, *et al.*, 2012).

The capacity of purified lysins as recombinant proteins to kill bacteria was first reported in 1959. Since then, research on phage lysins has highly evolved (Drulis-Kawa, *et al.*, 2012). However, only recently these enzymes were recognized as specific and potent antibacterial agents when added exogenously (Hermoso, *et al.*, 2007, Díez-Martínez, *et al.*, 2013). Although these enzymes, with high lytic activity, have been recognized for nearly a century, their use in a purified form did not receive much attention. However, with the advent of antibiotic-resistant pathogenic bacteria has sparked renewed interest and is now well established that these lysins, when added to bacteria in absence of bacteriophage, are able to irreversibly damage the bacterial cell wall (Hermoso, *et al.*, 2007, Díez-Martínez, *et al.*, 2013).

The phage lysins show numerous advantages when compared with the antibiotics: (1) hight specificity, they are usually specific to a species or subspecies (Hermoso, *et al.*,

2007), (2) low toxicity, (3) low probability of resistance development, and, (4) high efficiency (Drulis-Kawa, *et al.*, 2012).

Although lysins are generally strain specific, there are some cases of phage lysins with broad spectrum of lytic activity, for example the lysins PlyV12 and PlySs2 from bacteriophages of *Enterococcus faecalis* and *Streptococcus suis*, respectively (Díez-Martínez, *et al.*, 2013). Lysins encoded by Gram-negative specific phages normally exhibit a broad spectrum and are generally amidases. Several globular Gram-negative phage endolysins exhibiting broad spectrum activity have been described. Besides, endolysins exhibiting a broad spectrum may also be characteristic of some Gram-positive phages which encode amidase and non-amidase murein hydrolases (Drulis-Kawa, *et al.*, 2012).

1.2 Aquaculture

Aquaculture is the cultivation of aquatic populations, including fish, molluscs, crustaceans and plants, by individuals, groups or corporations that, under controlled conditions, such as controlled breeding, confinement, supplying nutrients and medicines, aim to increase their production (Sapkota, *et al.*, 2008). This form of culture of aquatic animals and plants exists in environments of fresh, brackish and marine water, either in open or closed systems (Pillay & Kutty, 2005).

The most frequent forms of aquaculture are open net pens or cages in offshore areas and ponds and tanks in coastal or inland waters (Almeida, *et al.*, 2009). The offshore aquaculture is often used to cultivate salmon, seabream, seabass, shrimp, catfish, trout, abalone, oysters and seaweed (Almeida, *et al.*, 2009, Menicou & Vassiliou, 2010).

Although more slowly than in the 1980s and 1990s, aquaculture production continues to grow in the new millennium, not only in terms of production but as a technological innovation to meet the demands of a growing world population. In 2010, world aquaculture production hit another high record of 60 million tons of fish. However, despite the growth in the international sector, the industry now faces some challenges to socio-economic, environmental and technological conditions. For example, the marine cage culture of Atlantic salmon in Chile, the oyster farming in Europe (particularly in France) and the marine shrimp farming in most countries of Asia, South America and Africa, had high mortality rates caused by disease outbreaks in recent years, resulting in

partial or sometimes total loss of production. Water pollution has increasingly threatened the production in some newly industrialized and rapidly urbanizing areas. In 2010, aquaculture in China suffered production losses of 1.7 million tons caused by diseases (295 000 tons), natural disasters (1.2 million tons) and pollution (123 000 tons). In 2011, disease outbreaks virtually eliminated the production of marine shrimp farming in Mozambique (FAO, 2012).

Microbial infections, namely bacterial infection, are the major concern in the aquaculture industry, leading to large financial losses endangering the sustainability of the sector (Almeida, *et al.*, 2009). The problem increases due to the emergence of multidrug resistant bacteria that infect a wide variety of fish (Almeida, *et al.*, 2009, Oliveira, *et al.*, 2012).

For these reasons, it is necessary and urgent to find new alternatives for effective control and treatment of microbial infections in aquaculture environments. Phage therapy is a promising new approach that can be used to reduce the impact of bacterial infections in aquaculture systems.

1.2.1 Fish farming diseases

Diseases are commonly found in farm fishes. The unfavorable conditions found in fish farms, such as the great density of fishes, increases the possibility of transference of pathogens between individuals. Due to high temperatures, rapid growth and overfeeding in fish cultivation, the conditions can become disadvantageous. With the accumulation of residues, a source of feeding for microorganisms, the risk of diseases outbreaks increases. Furthermore, when sick and dead fish are not extracted from the farming area in proper time, the risk of disease is higher, giving the opportunity to pathogens to become more aggressive when the environment is polluted. It was already been shown that pathogenic microorganisms are not introduced into the aquatic environment by farmed fish, although, it has been suggested that wild fish are a significant source of pathogenic microorganisms. Although the occurrence of sick individuals in wild stocks is generally low, the number of infected fish might be high, due to the fact that the individuals can be infected without exhibiting evident signs of disease (Weiss, 2002).

The main biological agents that cause waterborne diseases include bacteria, viruses, protists, helminths, oomycetes and fungi, however, various fish farming plants often suffer from heavy losses owing to frequent infections essentially caused by bacteria (Alderman, 1996, Wahli, *et al.*, 2002).

In terms of public health there are two groups of bacteria that infect fish with increased importance to the indigenous microflora (those naturally present in the environment) such as *Photobacterium damselae*, *Vibrio anguillarum*, *Vibrio vulnificus*, *Aeromonas hydrophila*, *Aeromonas salmonicida*, and non-indigenous microflora (those introduced by environmental contamination by excrement of domestic animals and/or human waste), like Enterobactereaceae such as *Salmonella* sp. and *Escherichia coli* (Muroga, et al., 1986, Huss, 1994, Nakai & Park, 2002).

Although a large number of bacteria cannot survive in environmental water and eventually die, a large number of these remains on the skin and in the gut of the fish being a risk to consumers health (Almeida, *et al.*, 2009).

The main diseases of marine and estuarine fish worldwide are vibriosis and photobacteriosis (formerly called pasteurellosis), both in natural production systems as well as in commercial ones, occurring only occasionally in freshwater fishes. Vibriosis and photobacteriosis diseases are responsible for major outbreaks in fish farm plants, reaching values of up to 100 % in the infected structures. Photobacteriosis and vibriosis are caused by bacteria of the Vibrionaceae family. Vibriosis is caused by species of *Photobacterium* (including *Photobacterium damselae* subsp. *Damselae*, formerly *Vibrio damselae*) and *Vibrio* (including *V. anguillarum*, *V. vulnificus*, *V. alfinolyticus*, *V. parahaemolyticus* and *V. salmonicida*). Photobacteriosis is caused by *P. damselae* subsp. *Piscicida* (formerly *Pasteurella piscicida*) which is a highly pathogenic bacterium that does not seem to have host specificity, infecting a wide range of fish species (Toranzo, *et al.*, 1991, Noya, *et al.*, 1995). In many countries of the Mediterranean such as France, Italy, Spain, Greece, Portugal, Turkey, Malta, Israel and Croatia, this pathogen causes high mortality rates in populations of seabass (*Dicentrarchus labrax*) and seabream (*Sparus aurata*) (Thyssen & Ollevier, 2001). Photobacteriosis continues to be a severe problem in intensive culture of different fish species in the Mediterranean area and Japan. Vibrionaceae species are also known to cause disease in humans and are mostly associated with the consumption of contaminated fish aquaculture. *Rickettsia*, *A. salmonicida* (causative agent of furunculosis)

and *Edwardsiella tarda* are also a significant group of fish pathogens, affecting a variety of fish species (Nakatsugawa, 1983, Mekuchi, *et al.*, 1995, Fryer & Lannan, 1996, Bernoth, 1997).

Viral diseases can also cause great losses in marine aquaculture (Munn, 2006, Saksida, *et al.*, 2006), however, the risk of infection in humans by the consumption of contaminated fish is low, because the viruses that cause diseases in fish are not pathogenic to humans (Almeida, *et al.*, 2009). Viruses that infect commercial fishes can be included in a variety of viral families, such as Irodovirus, Rhabdovirus, Birnavirus, Nodavirus, Reovirus, and Herpesviruses (Muroga, 2001, Suttle, 2007). The wide host range of these viruses and their ability to move between marine and fresh water, makes them a serious threat, allowing their spread into new areas (Meyers, *et al.*, 1999, Skall, *et al.*, 2005). Iridovirus has been identified as one of the most important pathogens, infecting more than 30 grouper species in the last decade (Wang & Wu, 2007). The viral nervous necrosis (VNN) disease caused by nervous necrosis virus is one important viral disease that causes mass mortality in more than 39 marine fish species of 10 families (Harikrishnan, *et al.*, 2011, Ma, *et al.*, 2012).

In respect to parasites, the main problem regarding food security focuses on a limited number of helminth species, and the risks are largely focused on communities where the consumption of raw or undercooked fish is a cultural habit. The main diseases transmittable to humans by the consumption of parasite contaminated fishes are trematodiasis, cestodiasis and nematodiasis. In addition to the hazards related to food safety, the parasites are also a concern in aquaculture because they often cause damage to the fish tissues, creating an ideal place to get a bacterial infection (Almeida, *et al.*, 2009).

In freshwater aquaculture, oomycetas are the second leading cause of infection in fish, after bacteria. Oomycetas may affect eggs, fingerlings and adult fishes when they are mechanically damaged or with infections caused by other pathogens (Ogbonna & Alabi, 1991, Gieseker, *et al.*, 2006). *Saprolegnia parasitica* is a very important fish pathogen, especially of catfish, salmon and trout species (Almeida, *et al.*, 2009). *Cryptocaryon irritans* is a parasite of commercial fishes (like grouper, for example) that is receiving worldwide attention. Besides the wide range of hosts, this parasite invades the skin, eyes and gills and causes the white spot disease in marine fish and is considered to be one of the most devastating parasite of marine fish (Harikrishnan, *et al.*, 2010).

1.2.2 Disease prevention in fish farms

A sustainable prevention of aquaculture diseases is desirable in health management, however, it is not always economically possible to supply optimal conditions and feeding ideal. The rapid spread and the ubiquitous nature of fish pathogenic microorganisms means that the prevention and control of infections is difficult (Almeida, *et al.*, 2009).

Vaccination would be the ideal method for the prevention of infectious diseases, however, the available vaccines in aquaculture are still very limited, and it is unknown whether vaccinated or disease resistant fish are able to clear viral pathogens when infected or are carrying it (Almeida, *et al.*, 2009, Johansen, *et al.*, 2011).

The vaccination basically works on the assumption that there is an immunological memory and that prior exposure to a pathogen allows a faster and stronger immune response (Lucas & Southgate, 2012). The success of vaccine development has been protecting the fishes against disease outbreaks and, in some cases, prevents or reduces the spread of pathogens from farmed to wild fish (Johansen, *et al.*, 2011).

Pasteurelosis and vibriosis have been largely controlled by the use of vaccines (Press & Lillehaug, 1995, Romalde, 2002). A vaccine against *Vibrio* spp. was effective in European Salmonid especially when administered by injection. Prophylactic immunization for other bacterial diseases in farmed fish has been tried with some success against *Aeromonas salmonicida* and *Yersinia ruckeri* (Press & Lillehaug, 1995). Vaccinated fish seem to grow and survive better than their unvaccinated counterparts, however, the exact nature of the immunity provided is unclear (Reed & Francis-Floyd, 1996). Despite some demonstrated successes, not all vaccines are effective enough and, for many pathogens, there are no available vaccines yet. For example, it is difficult to develop effective vaccines against intracellular bacteria (Johansen, *et al.*, 2011) and the vaccines in the field of aquaculture are still limited (Reed & Francis-Floyd, 1996., Romalde, 2002, Arijo, *et al.*, 2005, Lin, *et al.*, 2006). In addition, vaccination is not possible in the case of fish larvae, which are generally more susceptible to disease, since it is virtually impractical to deal with these small animals and because it is believed that the fish larvae do not have the ability to develop specific immunity (Vadstein, 1997, Almeida, *et al.*, 2009).

The development of vaccines for fish viral diseases, has been unsuccessful for a long period. Viral vaccines were developed for two viral diseases caused by the infectious

pancreatic necrosis virus and the infectious hematopoietic necrosis virus and a recombinant DNA-based vaccine was developed for the treatment of infectious pancreatic necrosis virus (Christie, 1997). However, these vaccines produced unsatisfactory results because the residual virulence killed the target species. In order to prevent the transmission of diseases and pathogens, it is necessary to understand the host-pathogen interactions and cell-mediated immune responses in fish (Johansen, *et al*., 2011).

The prevention and control of diseases in aquaculture becomes even more difficult due to several factors such as: (1) high levels of fecal contamination in fish farms water (poor water quality) (Huss, 1994, Howgate, *et al.*, 1997, Almeida, 2009); (2) irregular environmental conditions (i. e., elevated temperatures, salinity changes, decreased oxygen concentrations, high organic loads), factors that may contribute for the appearance of diseases, often weakened by the innate fish defense systems (Defoirdt, *et al*., 2007); (3) high densities (greater than what is indicated for each species) commonly used in fish farming systems, which reduce the resistance to infection (Defoirdt, *et al.*, 2007); (4) different stages of the fish life cycle, which increases the occurrence of infections (Defoirdt, *et al.*, 2007); (5) the indiscriminate use of antibiotics increased resistance problems in common pathogenic bacteria and increased the concern with the spread of antibiotics in the environment (Defoirdt, *et al*., 2007); (6) several chemotherapeutic agents that are effective against bacteria and oomycetes have low activity against endospores and zoospores (Alderman, 1999); (7) a significant number of pathogenic spores remain on the fish skin, even after quarantine (Alderman, 1999), and (8) few medications are licensed for use in fisheries (Kusuda & Kawai, 1998, Muroga, 2001).

1.2.3 Resistance to antibiotics

Although antibiotics were a viable and effective method for the treatment of diseases caused by pathogenic bacteria their indiscriminate and frequent use resulted in the development and spread of antibiotic resistance leads to ineffective treatment of bacterial infections (Defoirdt, *et al*., 2011).

The problem of antibiotic resistance becomes even more serious due to the fact that the spread of antimicrobial resistance is not necessarily restricted by phylogenetic, geographical and ecological borders. For this reason, the use of antibiotics in an ecological

niche, such as aquaculture, can lead to the occurrence of antimicrobial resistance in other ecological niches, including the human environment, making resistance to antibiotics a problem of public health concern (Heuer, *et al.*, 2009).

In marine environment, over 90% of the bacterial strains are resistant to more than one antibiotic, and 20% are resistant to at least five antibiotics (Martinez, 2003). The antibiotics are frequently ineffective in diseases treatments and a possible reason might be the fast replication of microorganisms that can induce quick mutations (Almeida, *et al.*, 2009). This possibility of mutation rapidly becomes prevalent throughout the microbial population, which helps the microbes to survive, for example in the presence of an antibiotic (Martinez, 2003). In aquaculture, the two most common *via* of antimicrobials administration are through the use of medicated feed or by the addition of antimicrobial agents directly into the water (immersion therapy) (Heuer, *et al.*, 2009).

The main problems in aquaculture systems of intensive and semi-intensive production arise from the regular use of artificial foods supplemented with antibiotics, in order to prevent the spread of diseases, which results in the development of resistant bacterial strains (Martinez, 2003).

In some cases, antibiotics are not effective in the treatment of bacterial diseases, as is the case of mass mortality in *Penaeus monodon* larvae caused by *Vibrio harveyi* strains with multiple resistance to cotrimoxazole, chloramphenicol, erythromycin and streptomycin (Karunasagar, *et al.*, 1994). Among these antibiotics, the first two were regularly used as prophylaxis (Cabello, 2006, Ishida, *et al.*, 2010).

Table 1.2 shows a panorama of the major classes of antibiotics used in the aquaculture industry and their importance in human medicine. Many antimicrobial agents used in human medicine are also used in aquaculture. In this way, the occurrence of resistance to these antibiotics in human pathogens severely limits the therapeutic options when treating infections in humans, and, therefore, the use of these antibiotics in animals must be controlled or prevented to avoid the spread of drug resistance. Among the antimicrobial agents commonly used in aquaculture, several are classified by the WHO (World Health Organization) as critically important for use in humans (Defoirdt, *et al.*, 2011).

Table 1.2 - Classes of antibiotics used in aquaculture, their importance for human medicine and examples of (multi)resistant pathogenic bacteria isolated from aquaculture (adapted from Defoirdt, *et al.*, 2011).

Drug class	Importance for human medicine[a]	Example	Resistant bacteria	Multiple[b] resistance	Isolated from
Aminoglycosides	Critically importante	Streptomycin	*Edwardsiella ictulari*	Yes	Diseased striped catfish (*Pangasianodon hypophthalmus*), Vietnam
Amphenicols	Important	Florfenicol	*Enterobacter* spp. and *Pseudomonas* spp.	Yes	Freshwater salmon farms, Chile
Beta-lactams	Critically importante	Amoxicillin	*Vibrio* spp., *Aeromonas* spp. and *Edwardsiella tarda*	Yes	Different aquaculture settings, Australia
Beta-lactams	Critically importante	Ampicillin	*Vibrio harveyi*	Yes	Shrimp farms and coastal waters,Indonesia
Fluoroquinolones	Critically importante	Enrofloxacin	*Tenacibaculum Maritimum*	Yes	Diseased turbot (*Scophthalmus maximus*) and sole (*Solea senegalensis*), Spainand Portugal
Macrolides	Critically importante	Erythromycin	*Salmonella* spp.	Yes	Marketed fish, China
Nitrofurans	Critically importante	Furazolidone	*Vibrio anguillarum*	Yes	Diseased sea bass and seabream, Greece
Nitrofurans	Important	Nitrofurantoin	*Vibrio harveyi*	Yes	Diseased penaeid shrimp, Taiwan
Quinolones	Critically importante	Oxolinic acid	*Aeromonas* spp., *Pseudomonas* spp. and *Vibrio* spp.	Yes	Pond water, pond sediment and tiger shrimp (*Penaeus monodon*), Philippines
Sulphonamides	Important	Sulphadiazine	*Aeromonas* spp.	Yes	Diseased katla (*Catla catla*), mrigel (*Cirrhinus mrigala*) and punti (*Puntius* spp.), índia
Tetracyclines	Highly importante	Tetracycline	*Aeromonas Hydrophila*	Yes	Water from mullet and tilapia farms, Egypt
Tetracyclines	Highly importante	Oxytetracycline	*Aeromonas Salmonicida*	Yes	Atlantic salmon (*Salmo salar*) culture facilities, Canada

[a] On the basis of World Health Organization Expert Consultations on 'Critically Important Antimicrobials for Human Medicine' (Heuer, *et al.*, 2009).
[b] Resistance to antibiotics belonging to different classes in at least one of the isolates.

About half of all antibiotics in the world are for animal use, like in fish farming, where antibiotics are used as growth promoters (Lorch, 1999, Almeida, *et al.*, 2009). In Asian countries a large variety of antibiotics is used. The equivalent of 500-600 tones per year are used prophylactically, some on a daily basis (Almeida, *et al.*, 2009).

In Thailand, in an interview with 76 shrimp producers, it was found that 56 used antibiotics (Holmström, *et al.*, 2003). In the Phillipines, Tendencia and Pena it was

reported the use of a large variety of antibiotics, namely oxytetracycline, oxolinic acid, chloramphenicol, furozolidine, nitrofurans, erythromycin and sulfonamids (Tendencia & de la Peña, 2001). In South America, a wide range of antibiotics is also used in aquaculture, including oxytetracycline, florfenicol, trimethoprim-sulfamethoxazole, enrofloxacin and sarafloxacin (Roque, *et al.*, 2001). In Europe, the use of antibiotics is also practiced in aquaculture, however, it is only allowed the use of a limited number of antibiotics, such as amoxacillin, ampicillin, chloramphenicol, erythromycin, florfenicol, flumequine, oxolinic acid, oxytetracycline, nitrofurazone, sulphadiazine-trimethoprim and tetracycline (Toranzo, *et al.*, 1991, Bakopoulos, *et al.*, 1995, Sano, 1998).

Despite the need for a stricter control on the use of antibiotics in aquaculture, to minimize or reduce the spread of antibiotic and its resistance, few countries monitor the amount of antibiotics used in aquaculture and, in this way, it is impossible to obtain a complete view of all the agents that are used in the aquatic system (Smith, 2008, Heuer, *et al.*, 2009). For example, recent studies made by Smith about the use of antibiotics reveal that there is a large variation between countries, ranging from 1 g per tonne of production in Norway to 700 g per tonne in Vietnam (Smith, 2008).

The use of antibiotics in aquaculture requires implementation of stricter regulations as well as regulations on the presence of antibiotic residues in aquaculture products (FAO, 2002). Although in some countries (like Japan and other countries in Europe and North America) there are already strict regulations on the use of antibiotics and only a few antibiotics are licensed for use in aquaculture, a large part of the world aquaculture production occurs in countries that have few or even no regulations (FAO, 2002, Smith, 2008).

When aquaculture products from countries that do not have adequate regulations are to be exported, they must follow the stricter regulations of the importing countries regarding the presence of antibiotic residues. In addition, all exporting countries have to fulfill the requirements of the Hazard Analysis and Critical Control Points (HACCP) for international trade (FAO, 2002).

Although the administration of antibiotics has been approved by the authorities, representing a low-cost and relatively easy application, this strategy has limited success when compared with the economic losses caused by bacterial infections (Oliveira, *et al.*, 2012).

Antibiotic resistance became, in recent years, a public health problem that must be solved as soon as possible. The solution for this problem passes by a difficult resolution that requires behavioural changes at various levels: at hospitals, with regard to medical behaviour in relation to the administration of antibiotics, and changes at the farm level and control of the use of these substances in animal exploration (Mellon, *et al.*, 2001).

Therefore, to reduce the risk of development and spread of microbial resistance and to control fish diseases in aquaculture, it is necessary to develop alternative strategies more environmentally friendly and economically viable (Almeida, *et al.*, 2009).

1.2.3.1 The use of antibiotics in aquaculture and the associated risks for human medicine

The use of antibiotics in aquaculture presents a risk to public health because of the potential development of antimicrobial resistance in fish pathogenic bacteria and in other aquatic bacteria. Antimicrobial resistance in fish pathogenic bacteria can act as reservoirs of resistance genes, from which genes can spread to human (Heuer, *et al.*, 2009, Defoirdt, *et al.*, 2011). For example, studies simulated in natural microenvironments indicate that the dissemination of resistance of R plasmids (plasmids harboring multiple antimicrobial-resistance determinants) can occur from *Aeromonas* species to *Escherichia coli* (Kruse & Sørum, 1994, Heuer, *et al.*, 2009). This form of spreading of antimicrobial resistance from aquatic environments to humans can be viewed as a form of indirect spread by horizontal gene transfer. Furthermore, some groups of aquatic bacteria (for example, some species of *Vibrio*) are considered as pathogens and other bacterial species may be opportunistic pathogens in humans. When a human infection is caused by bacteria of these groups, this form of antibiotic resistance dissemination is seen as direct transmission of antibiotic bacterial resistance from the aquatic environment (Heuer, *et al.*, 2009).

In addition to antibiotic resistant bacteria that represent a high risk to human health, the presence of antimicrobial residues in aquaculture products and the horizontal transference of genes also present a risk to humans and is often associated with allergies, toxicity, altered intestinal flora and selection of antimicrobial resistant bacteria (Paige, *et al.*, 1997, Cabello, 2006). However, the risk associated with antimicrobial residues depends on the type and amount of the antimicrobial agent found or consumed and,

generally, lower exposure means lower risk. Despite the risk associated with the ingestion of antimicrobial residues in food, toxicological effects of antimicrobial residues in food pose less risk to human health than the risk associated with antimicrobial resistant bacteria in foods (Kruse & Sørum, 1994). Furthermore, antibiotic-resistant bacteria also enter the marine environment by human or animal sources and have the ability to disseminate their resistance genes, increasing the problems associated with antibiotic resistant bacteria in the environment, such as, for example, the fish farms (Baquero, *et al.*, 2008).

The determining factors of antibiotic resistance that have emerged and/or evolved in the aquaculture environment are often located on mobile genetic elements. Resistant genes have been detected in transferable plasmids and integrons in pathogenic bacteria, such as *Aeromonas* spp., *Citrobacter* spp., *Edwardsiella* spp., *Photobacterium* spp. and *Vibrio* spp. (Ishida, *et al.*, 2010).

It has been well documented that fish pathogens and other aquatic bacteria can develop resistance as a consequence of exposure to antimicrobial agents. For example, a strain of *A. salmonicida* that causes disease in fish from temperate and cold climates, acquired resistance to sulphonamide and to quinolones. Resistance to quinolones in strains of *A. salmonicida* was mainly mediated by mutation in the gyrase A gene (*gyr*A) (Heuer, *et al.*, 2009). Resistance to norfloxacin, oxolinic acid, trimethoprim, and sulphamethoxasole was found in a local shrimp farming in Vietnam, and *Bacillus* and *Vibrio* species were predominant among bacteria that were resistant to these antimicrobials (Le, *et al.*, 2005). Resistance to sulphonamides in bacteria from shrimp hatcheries in India was also reported (Otta & Karunasagar, 2001). The problem of antibiotic resistance is even more concerning because some bacteria that are responsible for infections in fish belong to the same genera of the bacteria that cause infections in humans, increasing the probability of dissemination of antimicrobial resistance from aquaculture pathogens to human beings. For example, it has been demonstrated that plasmids harbouring the resistance determinants are transferable from fish pathogens and aquatic bacteria, not only to other bacteria of the same gender, but also to *E. coli* (Kruse & Sørum, 1994, Akinbowale, *et al.*, 2007). Plasmids carrying multiresistance determinants have shown to be transferable to *E. coli* from *A. salmonicida*, *A. hydrophila*, *E. tarda*, *Citrobacter freundii*, *P. damselae* subspecies *piscicida*, *V. anguillarum*, and *V. salmonicida* (Heuer, *et al.*, 2009). Plasmids transporting resistance to six antimicrobial agents were transferable from *Vibrio cholerae* O1 to *A.*

salmonicida, *A. hydrophila*, *Vibrio parahaemolyticus*, *V. cholerae*, *V. anguillarum*, *Shigella* species, *Salmonella* species and *E. coli* (Kruse, *et al.*, 1995). The genes that confer tetracycline resistance isolated in Japan in fish farm bacteria and human clinical bacteria showed high similarity, suggesting that they were derived from the same source (Furushita, *et al.*, 2003). In laboratory assays, tetracycline resistance from marine strains of *Photobacterium* species, *Vibrio* species, *Aeromonas* species and *Pseudomonas* species was transferred to *E. coli* by conjugation. The transference of plasmids containing resistant genes from fish pathogenic bacteria and other aquatic bacteria shows that these bacteria may act as reservoirs of resistance genes that can be further spread. Furthermore, molecular characterization of some resistance determinants indicates that antimicrobial resistance genes can be exchanged between fish pathogenic bacteria and human bacteria (Heuer, *et al.*, 2009).

Besides the indirect spread of antimicrobial resistance by horizontal gene transfer, dissemination of genes from aquatic antibiotic resistant bacteria to humans bacterial pathogens may also occur in a direct way (Heuer, *et al.*, 2009). Therefore, antimicrobial treatment of infections caused by these bacteria can result in failures in the treatment of infections in humans. The way of dissemination of antimicrobial resistance to humans can be by direct contact with water or aquatic organisms, through drinking water, or through handling or consumption of aquaculture products. Direct dissemination from aquatic environments to humans may involve human pathogens, such as *V. cholerae*, *V. parahaemolyticus*, *Vibrio vulnificus*, *Shigella* species, and *Salmonella* species, or opportunistic pathogens, such as *A. hydrophila*, *Plesiomonas shigelloides*, *E. tarda*, *Streptococcus iniae*, and *E. coli*. The occurrence of antibiotic resistance in *Salmonella* species in aquatic environments is probably attributed to contamination of human or animal origin, or from agricultural environments (Heinitz, *et al.*, 2000).

The transference of antibiotic resistance from aquaculture bacteria to humans has enormous consequences, namely the increasing of the number of infections in humans. This fact makes the treatment of microbial infections more difficult, because individuals that are in contact with an antimicrobial agent have a higher risk of infection with pathogens that are resistant to the same antimicrobial agent. Antibiotic resistance also increases the frequency of treatment failures, which may lead to an increased severity of the infection. The increase of treatment failures in humans, as a result of antibiotic

resistance, may result in a prolonged duration of the disease, resulting in a prolonged presence of the infection on the bloodstream, prolonged hospitalization, or increased mortality (Kruse & Sørum, 1994). For example, prolonged disease has been demonstrated in studies of fluoroquinolone resistant *Campylobacter* and for infections caused by quinolone-resistant *Salmonella typhimurium* an increased severity of the infection has been demonstrated (Smith, *et al.*, 1999, Neimann, *et al.*, 2003, Helms, *et al.*, 2004). Thus, the increasing resistance to antibiotics, including multirresistant bacteria, has become in recent years one public health problem, which has to be solved quickly. Without a solution, the treatment of bacterial infections will be more and more difficult. To solve this problem, more effective and environmentally friendly alternatives for the prevention and control of bacterial diseases are required.

1.3 Phage therapy: an alternative to antibiotics

Phage therapy is gaining more attention as a promising alternative to antibiotics and other antibacterial chemicals, to control and prevent bacterial diseases and to prevent the spread of multiresistant bacteria in aquaculture (Nakai & Park, 2002)**.**

Phage therapy involves the use of bacteriophages, lytic viruses that specifically inactive pathogenic bacteria, posing an effective alternative to antibiotics (Matsuzaki, *et al.*, 2005, Clark & March, 2006).

1.3.1 Application of phage therapy in aquaculture

The success of phage therapy in aquaculture involves a series of steps. Knowing the causative infectious agent it is subsequently possible to select the phage that may be able to efficiently infect the target bacteria, and thus, control the bacterial diseases in aquaculture. These steps include: (1) isolation of lytic bacteriophages from the fish environment, using a method of enrichment; (2) production of a phage stock; (3) genotypic and phenotypic characterization of the phage; (4) typing of bacteria and bacteriophage; (5) selection of a suitable lytic phage for therapeutic use; (6) evaluation of the therapeutic efficacy of the phage infections in laboratory and field trials, and (7) recognition of virulent genes or other toxic factors on the bacteriophage (Nakai, *et al.*, 2010).

The phage stocks to be used in aquaculture need to be prepared with some care, because these stocks should have high degree of purity. By this way, it is necessary to remove the bacterial debris (such as lipopolysaccharides and endotoxins), eliminating additional problems, since the contaminated phage suspensions can be fatal for the treated organism (Carlton, 1999, Inal, 2003, Efrony, *et al.*, 2007). For the success of phage therapy is fundamental to characterize and type the bacteriophages, due to the high degree of phenotypic and genotypic diversity within populations of both bacteria and phages (Stenholm, *et al.*, 2008). The preparation of a lytic phage to control the high diversity of pathogenic bacterial strains is critical because phages are generally strain specific. In this way, the preparation of cocktails with different lytic phages is a necessity (Nakai, *et al.*, 2010).

The efficiency of phage therapy should be evaluated not only *in vitro* (in laboratory experiments) but also in natural environments, because a phage may exhibit lytic in properties in experimental environments but not *in vivo* (Sandeep, 2006). Furthermore, field assays also aim to establish the dose and route of phage administration. Concerning the treatment of fish with phage, the available data about the doses of phages and effective routes of administration are limited. However, in contrast to chemicals and other substances, a precise determination of the initial dose of the phage given to each fish may not be essential in aquaculture because the phages are able to increase along with the bacteria (Nakai, *et al.*, 2010).

Phage administration may be by injection, addition to culture water or impregnated feed (Nakai & Park, 2002). Even considering that the diseased fish cannot get sick eating so well, phage by impregnated feed allows the treatment of a large number of fish specimens (Nakai et al, 2010). This technique can be advantageous for infections that occur orally, since the bowel is also a route for the pathogen into the organism, and normal intestinal flora might be unaffected, but the target bacteria will be. Although treatment with the phage can also be accomplished by injection, it can be laborious when a large number of animals needs treatment or when dealing with very small animals. However, this approach is used for a series of vaccines on the market (Nakai, *et al.*, 2010). The addition of phages in the culture water is also possible because these tend to remain stable and effective as if in a liquid culture medium (Nakai, *et al.*, 1999). This type of administration has the advantage of being continuous and facilitates the physiological contact between the

infected organism and the bacteriophage (Summers, 2001, Inal, 2003). For this reason, the administration of therapeutic phages in water (immersion) will be more effective to organisms in which the infection is initiated by bacterial colonization of the skin and gills (Nakai & Park, 2002). Additionally, in terms of comparison of results between the laboratory and the field, the immersion approach allows greater similarity between environmental and laboratory conditions because the phage-bacteria interaction occurs in suspension (Summers, 2001). Treatment of larvae, juveniles or hatcheries eggs by the bathing or immersion techniques has been efficient, for example, in the biocontrol of *Vibrio harveyi* in *Penaeus monodon* larvae (Vinod, *et al.*, 2006, Karunasagar, *et al.*, 2007). In aquaculture, the possibility of using multiple routes of phage administration is very advantageous since microbial infections may occur in various stages, from eggs to broodstock (Nakai, *et al.*, 2010).

Regarding the phages immune response , they are recognized by the immune system of animals as external entities, and hence an immune response can be developed (Pirisi, 2000, Sulakvelidze & Morris, 2001). Phage neutralizing antibodies may decrease phages efficacy *in vivo* so, for this reason, a higher dose of phage may be required in order to compensate non-viable phages that are processed by interaction with neutralizing antibodies (Carlton, 1999, Pirisi, 2000, Sulakvelidze & Morris, 2001). In order to overcome immune responses, it is important to test whether phage neutralizing antibodies are produced and for how long they remain in circulation, which factors of the vertebrate host immune response are capable of inactivating the phage and if the phage inoculations given too early may be less effective (Barrow & Soothill, 1997, Yuksel, *et al.*, 2001, Payne & Jansen, 2003). However, the production of phage neutralizing antibodies after administration of the phage in aquaculture is not documented in the literature (Nakai, *et al.*, 1999, Oliveira, *et al.*, 2012). The immune response is not a problem for phage therapy in invertebrate hosts, and this approach has proven effective in these organisms (Efrony, *et al.*, 2007).

1.4 Advantages of phage therapy over chemotherapy in fish farms

Phage therapy has many advantages when compared to chemotherapy: (1) it is effective against multi-resistant pathogenic bacteria because the mechanisms by which it induces the lysis of the bacteria are completely different from that of antibiotics. Bacteria will certainly develop resistance to the phage but, since the phage has a greater rate of mutation and replication it can adapt to the development of resistant bacteria and so the development of resistance is limited (Matsuzaki, *et al.*, 2003, Matsuzaki, *et al.*, 2005). The fact that the phage co-evolves with its bacterial host makes the discovery of new phages relatively easy when compared to the development of new antibiotics. In addition, bacteria that become resistant to a phage are infected by new phage mutants (Matsuzaki, *et al.*, 2003). Another possibility to reduce the appearance of resistant strains during treatment with the phage preparation is to make a mixture of different phage strains (Biswas, *et al.*, 2002, Watanabe, *et al.*, 2007). Moreover, phage resistant bacteria are still not necessarily pathogenic because selection for resistance could select against virulence (Levin & Bull, 2004, Sandeep, 2006, Nakai, *et al.*, 2010); (2) The phages are usually highly specific to a single bacterial species or strain and therefore cause much less damage to the intestinal flora of normal fish and other non-target bacterial species (Matsuzaki, *et al.*, 2003); (3) The phages are self-replicating and self-limiting, unlike antibiotics and thus have a limited impact. They replicate exponentially along with bacteria and decrease when the number of bacteria decreases, without any environmental risk. The number of times that the phage must be applied varies with the application, however, a single dose may be sufficient, and the determination of the precise initial dose may not be essential since titers may increase with bacterial infection (Inal, 2003, Mathur, *et al.*, 2003); (4) The approval and regulation of the treatment with phages can be substantially simple since naturally occurring phages are very abundant; (5) The phages are resistant to environmental conditions, whether the phages are found in the same environment as the bacterial host or not, the capacity of these phages to survive in the same environment as their host bacteria is high (Durán, *et al.*, 2002, Lucena, *et al.*, 2004); (6) The development of phage therapy is more cost-effective than the development of a new antibiotic and is faster and more flexible (Carlton, 1999, Matsuzaki, *et al.*, 2005).

1.4.1 Phage cocktails in the treatment of bacterial infections

Despite the proven success of phage therapy with just one phage, being this form of therapy effective when the pathogenic bacterial strain is known and clearly defined, there are certain factors that can lead to the failure of phage therapy when one phage alone is used. These factors are the development of phage resistance to the pathogenic bacteria and the narrow host range of the phages, which makes the control of bacterial species criticized because they are often strain specific phages. However, the use of phage cocktails circumvents these problems (Gill & Hyman, 2010, Nakai, *et al.*, 2010).

In phage infection, the attachment of the phage to the bacterium is one essential step. For this step to occur, the phage can use bacterial capsules, different parts of lipopolysaccharide (LPS), flagella, fimbriae and many other surface proteins as receptors. Furthermore, the phage may also use enzymes to break down capsule like materials on the bacterial surface, in a drill-like manner, to reach the cell wall of the bacterium (Skurnik & Strauch, 2006). The resistance of bacterial strains to lytic phages is partly due to mutations or by loss of the phage receptor (Shivu, *et al.*, 2007).

Bacteria that are phage resistant are not necessarily as pathogenic as the wild type because selection for resistance can be accompanied by a decrease in virulence. Because bacterial mutations can occur in the phage receptor, they can also act as a virulent factor (Merril, *et al.*, 2006). In addition, the bacterium may become phage-resistant also by lysogeny conversion, whereby the bacterium becomes immune to the phage and to its close relatives (Skurnik & Strauch, 2006).

The success of phage cocktails in the particular case of bacterial resistance requires that the selected phages do not possess overlapping cross-resistance, that is, bacterial mutants that are resistant to one phage are still sensitive to the other, and *vice versa*. Thus, cocktails containing only phages with LPS receptors can theoretically be less successful than a phage cocktail containing phages using different receptors. It is also needed that bacteria can be infected by several phages (Gill & Hyman, 2010). A concern regarding the use of phage cocktails is related to the ability of the phages to interfere with each other by co-infection. LMP-102 made by Intralytix Incorporated (Baltimore, MD, USA) is a successful example of the use of a phage cocktail to limit the development of host resistance. This product consists of a mixture of six bacteriophages infecting several strains

of *L. monocytogenes*, a common contaminant of fresh meat and meat products (Abedon & Thomas-Abedon, 2010).

In the treatment of certain human diseases, when the pathogenic bacterial strain is not known and prompt treatment is required, the use of phage cocktails simplify the hired treatments by the use of individual phages (Dowd, *et al.*, 2008, Abedon & Thomas-Abedon, 2010). The need for phage cocktails is evident in the treatment of certain wound infections, where it is increasingly understood that these infections are due to polymicrobial communities instead of bacterial monocultures (Dowd, *et al.*, 2008). Thus cocktails may serve as a first line treatment, followed by specific correspondence between phages and bacteria, if required (Gill & Hyman, 2010).

The narrow host range of phages is pointed out as a drawback commonly listed in phage therapy when compared with antibiotics, however, the use of cocktails circumvents this disadvantage (Gill & Hyman, 2010). The commercial product PhagoBioDerm contains a variety of phages which are capable of lysing *E. coli*, *Pseudomonas aeruginosa*, *S. aureus*, *Proteus* and *Streptococcus* (Markoishvili, et al., 2002, Kutter, et al., 2010).

To evaluate the efficacy of bacteriophages in the treatment of burn wound infections, one phage cocktail (BFC-1) consisting of three phages, a Myovirus and a Podovirus against *P. aeruginosa* and a Myovirus against *S. aureus* was prepared. BFC-1 was applied by a single spray application in the burn wound. One part of the wound was not treated with the phages and was used as a control. The patients were monitored for three weeks after the treatment and it were not observed any type of clinical abnormalities that could be related to the application of phages and the infection was controlled (Merabishvili, *et al.*, 2009, Kutter, *et al.*, 2010).

In aquaculture, the studies show contradictory results with regard to the appearance of phage-resistant bacteria. Some studies indicate bacterial resistance to phage as a consequence of phage therapy in diseased fish or in apparently healthy fish, but others do not show this effect (Park & Nakai, 2003). The therapeutic effects against infection in ayu were examined. In this study, the *in vivo* efficacy of the PPpW-4 (Podoviridae), PPpW-3 (Myoviridae) and a mixture of both phages (PPpW-3/W-4) was evaluated against *Pseudomonas plecoglossicida* infection. The results show that the phage cocktail (PPp-W4/ PPp-W3) exhibited high inhibitory activity when compared with the phage alone. This inhibitory activity shows that no phage-resistant organisms nor phage neutralizing

antibodies were detected in diseased fish or in apparently healthy fish, showing that the treatment with bacteriophages can be a potential alternative for the control of diseases (Park & Nakai, 2003).

In addition to the potential use of phage cocktails for the treatment of fish diseases, the mixture of lytic bacteriophages for the biocontrol of food-borne pathogens in food industry is gaining increasing acceptance. In a recent study, the ability of a cocktail of five phages infecting *Vibrio* for the treatment of *Vibrio* cholera O1 in a rabbit model was examined. The phage cocktail (10^8 plaque forming unit), in one group of rabbits, was administrated 6 and 12 h prior to the administration of *V. cholerae* O1, while in the other group the same procedure was applied 6 and 12 h post infection. The results show that oral administration of the phage cocktail after oral bacterial challenge significantly lowered the shedding of bacteria, and in contrast, the phage treatment prior to bacterial challenge had no such effect. These results indicate that oral administration of the phage subsequent to *V. cholerae* challenge could provide a possible mean for combatting *V. cholerae* infection. Furthermore, the results showed that the phage cocktail could not only reduce the *V. cholera*e counts more rapidly when compared to the individual phages but also to a greater extent, indicating the possible usefulness of the phage cocktails as a therapeutic agent for combatting *V. cholera*e infection (Jaiswal, *et al.*, 2013). Other study made with a phage cocktail composed of three different lytic bacteriophages (UAB_Phi 20, UAB_Phi78, and UAB_Phi87) was used in four different food matrices (pig skin, chicken breasts, fresh eggs and packaged lettuce) experimentally contaminated with *Salmonella enterica* serovar *Typhimurium* and *S. enterica* serovar *Enteritidis*. When pig skin was sprayed with the bacteriophage cocktail and then incubated at 33 ºC for 6 h, a significant reduction of *Salmonella* was obtained (> 4 and 2 log/cm^2 for *S. typhimurium* and *S. enteritidis*, respectively). A significant bacterial reduction in the concentration of *S. typhimurium* and *S. enteritidis* was also observed in chicken breasts dipped for 5 min in a solution containing the bacteriophage cocktail and then refrigerated at 4 °C for 7 days (2.2 and 0.9 $\log_{10}$ CFU/g, respectively) as well as in lettuce treated for 60 min at room temperature (3.9 and 2.2 $\log_{10}$ CFU/g, respectively). However, in fresh eggs sprayed with the bacteriophage cocktail and then incubated at 25 ºC for 2 h, only a minor reduction of bacterial concentration (0.9 log10 CFU/cm^2 of *S. enteritidis* and *S. typhimurium*) was achieved. These results demonstrate the potential efficacy of this phage cocktail as a biocontrol agent

for *Salmonella* in several food matrices, under conditions similar to those used in their production (Spricigo, *et al.*, 2013).

1.5 Objectives of the work

Although several bacteriophages related to major aquaculture diseases have already been identified and characterized (Merino, *et al.*, 1990, Myhr, *et al.*, 1991, Munro, *et al.*, 2003, Martinez-Urtaza, *et al.*, 2004), the application of phage therapy against bacterial diseases in aquaculture is not used as a routine. The main purpose of this work was to improve the phage therapy protocols in order to their effective use in aquaculture.

The specific objectives were:

- To test the effect of phage cocktails in the treatment of bacterial infections in aquaculture, particularly in the treatment of vibriosis.

- To evaluate the effect of lysozyme addition on the activity of the phages.

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Chapter 2: Efficiency of phage cocktails in the inactivation of *Vibrio* in aquaculture

[In press. Aquaculture.]

Abstract

Aquaculture is one of the fastest growing sectors in the world, having a very important role in the economy. However, the losses associated with bacterial infections, such as vibriosis lead to huge economic costs. The regular use of antibiotics in aquaculture has resulted in the development of resistant strains, which have contributed to the inefficacy of antibiotics. To reduce the risk of the development and spreading of microbial resistance and to control the fish diseases in aquaculture, alternative strategies must be developed. Phage therapy can be an eco-friendly alternative to prevent and control pathogenic bacteria in aquaculture. However, phage bacterial resistance is already well documented but the use of phage cocktails can overcome this drawback.

The aim of this study was to evaluate the efficiency of cocktails of two and three phages of *Vibrio parahaemolyticus* (VP-1, VP-2 and VP-3) to control *Vibrio* in aquaculture. All phages were effective against *V. parahaemolyticus*, however, the VP-3 phage was the most efficient one (additional reduction of more 2 log when compared with the other two phages). The use of cocktails with two and three phages was significantly more effective (reductions of 4 log after 2 h) than the use of VP-1 and VP-2 phages alone (reductions of 0.8 log after 2 h), however, the efficiency of VP-3 phage was similar for the phage alone and for the phage in the cocktails (reduction of 3.8 log and 4.2 log for VP-3 phage alone and in cocktails, respectively, after 8 and 6 h). All phages remained viable for a long time (at least 5 - 7 months) in marine water. The VP-3 phage presented a larger burst size and a shorter latent period (42 and 40 min, respectively) than the other two phages (9 and 120 min and 15 and 90 min, respectively, for VP-1 and VP-2 phages). Overall, the use of phage cocktails of two or three phages increased the efficiency of phage therapy against *Vibrio* (more efficient and faster bacterial inactivation), delaying the development of resistance by the bacteria and the use of *Vibrio* phages with high burst sizes and short lytic cycles also increases the efficiency of phage therapy.

Keywords: Phage therapy, pathogenic bacteria, vibriosis, phage cocktails, aquaculture system.

Introduction

In commercial aquaculture, unfavorable conditions such as: overfeeding, high temperature, fast growth, infrequent water renewal rate and improper removal of wounded and dead animals from the farming area, create favorable conditions for the emergence of bacterial diseases (Almeida, *et al*., 2009).

Bacterial infections, including multidrug-resistant strains, have been recognized as an important limitation to the development of the aquaculture production (FAO, 2009, Oliveira, *et al*., 2012). Vibriosis, caused by bacteria from the family Vibrionaceae, are currently responsible for most outbreaks in aquaculture. Vibriosis is caused by species of *Vibrio* (namely *V. parahaemolyticus*, *V. anguillarum*, *V. vulnificus*, *V. alfinolyticus* and *V. salmonicida*) (Toranzo, *et al*., 1991, Hanna, *et al*.; 1992, Benediktsdottir, *et al*., 1998, Noya, *et al*., 1995, Sung, *et al*., 1999, Almeida, *et al*., 2009, Silva-Aciares, *et al*., 2013).

V. parahaemolyticus is an important human bacterial pathogen that is widely distributed in marine environments, frequently isolated from a variety of seafood including codfish, sardine, mackerel, flounder, clam, octopus, shrimp, crab, lobster, crawfish, scallop and oyster (Liston, 1990; Su & Liu, 2007). This bacterium of marine environments is frequently associated with the development of acute gastroenteritis in human by consumption of raw or undercooked contaminated seafood, particularly shellfish (Kaysner & DePaola Jr, 2000). The regular use of artificial food supplemented with antibiotics in intensive and semi-intensive aquaculture systems, to prevent the spread of diseases and their massive use to control infections, has resulted in the development of resistant strains, which have contributed to the inefficacy of antibiotic treatments (Martinez, 2003). To reduce the risk of the development and spreading of microbial resistance and to control the fish diseases in aquaculture, alternative strategies must be developed (Defoirdt, *et al*., 2011). Phage therapy can be used as an alternative to prevent and control pathogenic bacteria in aquaculture.

A major concern regarding the use of phages in the treatment of infectious diseases is the emergence of phage-resistant mutants (Smith & Huggins, 1983; Gil & Hyman, 2010).

Resistance may arise due to the alteration or loss of the bacterial cell surface receptors, blocking of the receptors by the bacterial extracellular matrix, inhibition of phage DNA penetration, production of modified restriction endonucleases that degrade the phage DNA, or due to the inhibition of the phage intracellular development (Labrie, *et al*., 2010). Mutations affecting phage receptors represent the most frequent cause of bacterial phage resistance (Heller, 1992; Labrie, *et al*., 2010).

Although the development of phage-resistance, when only one phage is used, has already been reported (Levin & Bull, 2004, Tanji, *et al*., 2005, Merril, *et al*., 2006, Sandeep, 2006, Skurnik & Strauch, 2006, Scott, *et al*., 2007, Nakai, *et al*., 2010, Vieira, *et al*., 2012; Silva, *et al*., 2013), this limitation can be overcame by the combined use of more than one phage at the same time, that is, by the use of phage cocktails (Crothers-Stomps, *et al*., 2010, Chan, *et al*., 2013). Furthermore, previous reports suggested that virulent bacteria that are resistant to phage infection could be less fit or could lose their pathogenic properties (Anonymus, 1983; Capparelli, *et al*., 2010, Fillippov, *et al*., 2011). Bacterial cell surface components that act as receptors for phage adsorption can also act as virulent factors, which may undertake mutation when bacteria develop resistance to phages, rendering them not pathogenic.

Phage cocktails not only potentially provide a means to circumvent resistance to the presence of a single phage but they also allow the treatment of multiple pathogens simultaneously (Cairns, *et al*., 2009, Merabishvili, *et al*., 2009, Kunisaki, 2010). Therefore, the high specificity of bacteriophages, that sometimes can be considered to be a disadvantage of phage therapy, namely when the photogenic bacteria are not known, may be circumvented by the use of phage cocktails, which broaden the spectrum of action (Sulakvelidze, *et al*., 2001, Chan, *et al*., 2013).

The aim of the present study was to test the efficacy of phage cocktails of two and three *V. parahaemolyticus* phages (VP-1, VP-2 and VP-3 phages) to control *V. parahaemolyticus* in aquaculture systems. The phages were tested alone and combined as cocktails. As the selection of appropriate bacteriophages is a key factor in the success of phage mediated-control of aquaculture infections, the three phages were characterized in terms of survival in the marine environment, host range, latent period and burst size.

Materials and methods

Bacterial strains and growth conditions

The bacterial strains, *V. parahaemolyticus*, *V. anguillarum*, *A. salmonicida*, *P. aeruginosa*, *P. fluorescens*, *P. putida*, *P. segetis* and *P. gingeri*, used in this study were previously isolated in our laboratory (Pereira, *et al*., 2011, Louvado, *et al*., 2012). The other bacterial strains, *P. damselae* subsp. *damselae* (ATCC 33539), *E. coli* (ATCC 13706), *V. fischeri* (ATCC 49387), *A. hydrophilla* (ATCC 7966), were purchased from ATCC collection. Fresh plate bacteria cultures were maintained in solid Tryptic Soy Agar medium (TSA; Merck, Darmstadt, Germany) at 4 °C. Before each assay, one isolated colony was aseptically transferred to 10 mL of Tryptic Soy Broth medium (TSB; Merck, Darmstadt, Germany) and was grown overnight at 25 °C. An aliquot of this culture (100 µL) was aseptically transferred to 10 mL of fresh TSB medium and grown overnight at 25 °C to reach an optical density (O.D. 600) of 0.8, corresponding to about 10^9 cells per mL.

Phages isolation and purification

Three phages (VP-1, VP-2 and VP-3) were isolated from marine water samples (salinity 18-21; pH 7.6-7.7) of a semi-intensive aquaculture (earthen pond aquaculture system Corte das Freiras located in the estuarine system Ria de Aveiro, latitude: 40º37´51.44´´N, longitude 8º40´31.75´´W, on the north-westem coast of Portugal) using *V. parahaemolyticus* as host. Five hundred milliliters of water were filtered sequentially by 3 µm and then by 0.45 µm pore-size polycarbonate membranes (Millipore, Bedford, Billerica, USA). Filtered water was added to 500 mL of TSB with double concentration and to 1 mL of bacterial culture. The mixture was incubated at 25 ºC for 18 h at 80 rpm, and then filtered through a 0.45 µm membrane. The presence/absence of the bacteriophage was verified through the spot test (Vieira, *et al.* 2012). Thirty microliters of the resulting filtrate were inoculated into TSA growth medium previously inoculated with the bacterial culture. The plates were incubated at 37 ºC for 4 - 12 h and inspected for zones of clearing. Three successive single-plaque isolations were performed to obtain a pure phage stock. All lysates were centrifuged at 10.000 g during 10 min at 4 °C, to remove intact bacteria and

bacterial debris. The phage stocks were stored at 4 °C and were added of 1% chloroform (Scharlau, Spain). The phage suspension titer was determined by the double-layer agar method using TSA as culture medium (Adams, 1959). The plates were incubated at 37 ºC for 4 - 8 h and the number of plaques was counted. The results were expressed as plaque forming units per millilitre (PFU mL^{-1}).

Host range determination and efficiency of phage infection

To determine the phage host specificity, the double-layer agar method was used. Spot test was performed with twelve bacteria (*V. parahaemolyticus*, *A. salmonicida*, *V. anguillarum*, *P. damselae subsp. damselae*, *E. coli* (ATCC 13706), *V. fischeri* (ATCC 49387), *A. hydrophilla*, *P. aeruginosa*, *P. fluorescens*, *P. putida*, *P. segetis* and *P. gingeri*) to assess the bacterial susceptibility to the bacteriophage (Vieira, *et al.* 2012). The plates were incubated at 37 ºC for 4 - 12 h. The efficiency of plating was determined for the bacteria with positive spot tests (occurrence of a lysis zone) by the double-layer agar method using TSA as culture medium. Efficiency of plating for each host was calculated by comparison with an efficiency of 100% for the *V. parahaemolyticus* bacterium. For each phage, three independent experiments were done and the results were presented as the average of the three assays.

Phage survival determination

The survival of *V. parahaemolyticus* phages was tested in marine water of the aquaculture system Corte das Freiras, in three different dates, between May and July 2013. In each date, 50 mL of water were filtered through 0.45 μm and then by 0.22 μm pore-size membranes (Poretics, USA) which was followed by the addition of phage suspensions of about 10^7 PFU mL^{-1}. The samples were then incubated at 25 °C without shaking, in the dark. Phage titer was determined at time zero and at intervals of 12 h until the first day, 24 h until the fifth day, 48 h until the ninth day, 72 h until the twelfth day, 120 h until the forty-fifth day and 240 h until the end of the experiment (225 days), by the double-layer agar method. For each phage, three independent experiments were done.

One step growth assays

Mid-exponential host bacterial cultures of *V. parahaemolyticus* were adjusted to a 1 O.D. at 600 nm (corresponding to a cell density of 10^9 CFU mL^{-1}). Ten microliters of the phage suspension were added to 10 mL of the bacterial culture in order to have a multiplicity of infection (MOI) of 0.001. The phage was allowed to adsorb for 5 min at 25 ºC, without shaking. The mixture was centrifuged at 13.000 rpm for 5 min, the pellet was re-suspended in 10 mL of TSB at 37 ºC and was then serially diluted to 10^{-4}. Samples (1 mL) were taken at 10 - 20 min intervals and subjected to phage titration by the double-layer agar method. Three independent assays were done.

Phage therapy assays

Phage therapy was performed using one phage alone (VP-1, VP-2 or VP-3) and with phage cocktails (two or all the three phages mixed together, each phage at the same concentration) using the bacterium *V. parahaemolyticus* as host, at a MOI of 100. The assays with two phages were performed with the following phage combinations: VP-1/ VP-2, VP-1/VP-3 and VP-2/VP-3 phages. After that, phage therapy was performed with the combination of the three phages (VP-1, VP-2 and VP-3). In order to obtain a MOI of 100, 20 µL of the overnight *V. parahaemolyticus* culture (10^5 CFU mL^{-1}) and 300 µL of the phage suspension (10^7 PFU mL^{-1}), were inoculated into sterilized glass erlenmeyers with 30 mL of TSB medium and incubated at 25 ºC without agitation in the dark (test samples).

When the assays were performed with the cocktail of two and three phages, the same concentration of each phage was added (100 µL of each phage at 10^7 PFU mL^{-1}). For each assay, three control samples were included: the bacterial control (BC), the phage control and the phage cocktails controls (PC). The bacterial control was not inoculated with the phages and the phage controls were inoculated with the phage(s) but without the bacteria. All controls were incubated exactly in the same conditions as the test samples. Aliquots of test samples and of the bacterial and phage controls were collected after 0, 2, 4, 6, 8, 10, 12, 18 and 24 h of incubation. For all phage therapy assays, the phage titer was determined, in duplicate, by the double agar layer method, after an incubation period of 4 - 12 h at 25

ºC. The bacteria concentration was determined, in duplicate, in TSA medium after an incubation period of 24 h at 25 ºC. Three independent experiments were performed for each condition.

Statistical analysis

Statistical analysis was performed using SPSS (SPSS 20.0 for Windows, SPSS Inc., USA). Normal distributions were checked by Shapiro-Wilk test and homogeneity of variances by Levene test. The existence of significant differences among the different phage therapy conditions was assessed by one-way analysis of variance (ANOVA) model with the Tukey post-hoc test. For each situation, the significance of the differences was done by comparing the results obtained in the test samples after correction with the results obtained for the correspondent control samples (difference between in the control and the test sample) for the different times of each of the three independent assays. A value of $p < 0.05$ was considered to be statistically significant.

Results

Phages host range determination

The VP-1 phage infected *V. anguillarum* and *A. salmonicida*, presenting an efficiency of 83.27 and 64.75%, respectively (Table 2.1). The VP-2 phage infected *V. anguillarum* and *A. salmonicida* with an efficiency of 93.39 and 92.03%, respectively (Table 2.1). The VP-3 phage also infected *V. anguillarum* and *A. salmonicida*, presenting an efficiency of 51.21 and 73.78%, respectively (Table 2.1). None of the three phages was effective against *A. hydrophilla*, *P. damselae* subsp. *damselae*, *V. fischeri*, *E. coli*, *P. aeruginosa*, *P. fluorescens*, *P. putida*, *P. gingeri* and *P. segetis*.

Table 2.1- Efficiency of plating (%) to different bacteria.

Fish bacteria	Efficiency of phage plating (%)		
	VP-1	**VP-2**	**VP-3**
V. parahemolyticus	100	100	100
V. anguillarum	83.27	93.39	51.21
A. salmonicida	64.75	92.03	73.78
A. hydrophilla	0	0	0
V. fischeri	0	0	0
P. damselae subsp. *Damselae*	0	0	0
E. coli	0	0	0
P. aeruginosa	0	0	0
P. fluorescens	0	0	0
P. putida	0	0	0
P. segetis	0	0	0
P. gingeri	0	0	0

Phage therapy assays

Phage therapy using single-phage suspensions

The maximum of bacterium inactivation with the VP-1 phage was 2.9 log achieved after 6 h of phage therapy (ANOVA, $p < 0.05$). After 12 h of phage therapy, the rate of inactivation (bacterial inactivation relatively to the bacterial control at each time) was still considerably high (2.1 log) (ANOVA, $p < 0.05$) (Figure 2.1A). No decrease of the phage survival was observed during the 24 h of the experiments, for the VP-1 phage alone and for the phage in the presence of its host *V. parahaemolyticus*. While the phage control remained almost constant during all experiment (ANOVA, $p > 0.05$), when the phage was

incubated in the presence of its host, a significant increase of 0.7 log was observed after the 24 h (ANOVA, $p < 0.05$) (Figure2.1B).

With the VP-2 phage, the maximum of bacterium inactivation was 3.6 log, achieved after 8 h of phage therapy (ANOVA, $p < 0.05$). However, after 6 h of incubation, the rate of inactivation was already 3.23 log (ANOVA, $p < 0.05$). After 12 h of treatment, the rate of inactivation was still considerably high (2.8 log) (ANOVA, $p < 0.05$) (Figure 2.1A). The phage control remained almost constant during all time (ANOVA, $p > 0.05$), but when the phage was incubated in the presence of its host, a significant increase of 0.7 log (ANOVA, $p < 0.05$) was observed after 24 h of phage therapy (Figure 2.1B).

The maximum of bacterium inactivation by the VP-3 phage was 3.8 log, achieved after 8 h of phage therapy (ANOVA, $p < 0.05$). However, soon after 2 h of phage therapy, the rate of inactivation was already 2.6 log (ANOVA, $p < 0.05$). After 12 h of incubation, the rate of bacterium inactivation was still considerably high (2.1 log) (ANOVA, $p < 0.05$) (Figure 2.1A). The phage control remained almost constant during all experiment (ANOVA, $p > 0.05$) but the concentration of the phage incubated in the presence of its host, significantly increased (increase of 0.30 log) after the 24 h (ANOVA, $p < 0.05$) of phage therapy (Figure 2.1B).

The rate of bacterial inactivation with the VP-3 phage (maximum reduction of 3.8 log after 8 h) was, in general, significantly higher (ANOVA, $p < 0.05$) than those obtained with the VP-1 and VP-2 phages. The bacterial inactivation of VP-3 also started sooner, after 2 h, and the rate of inactivation (2.6 log) was significantly higher (ANOVA, $p < 0.05$) than those of VP-1 and VP-2 phages (0.6 and 0.8 log, respectively) (Figure 2.1A).

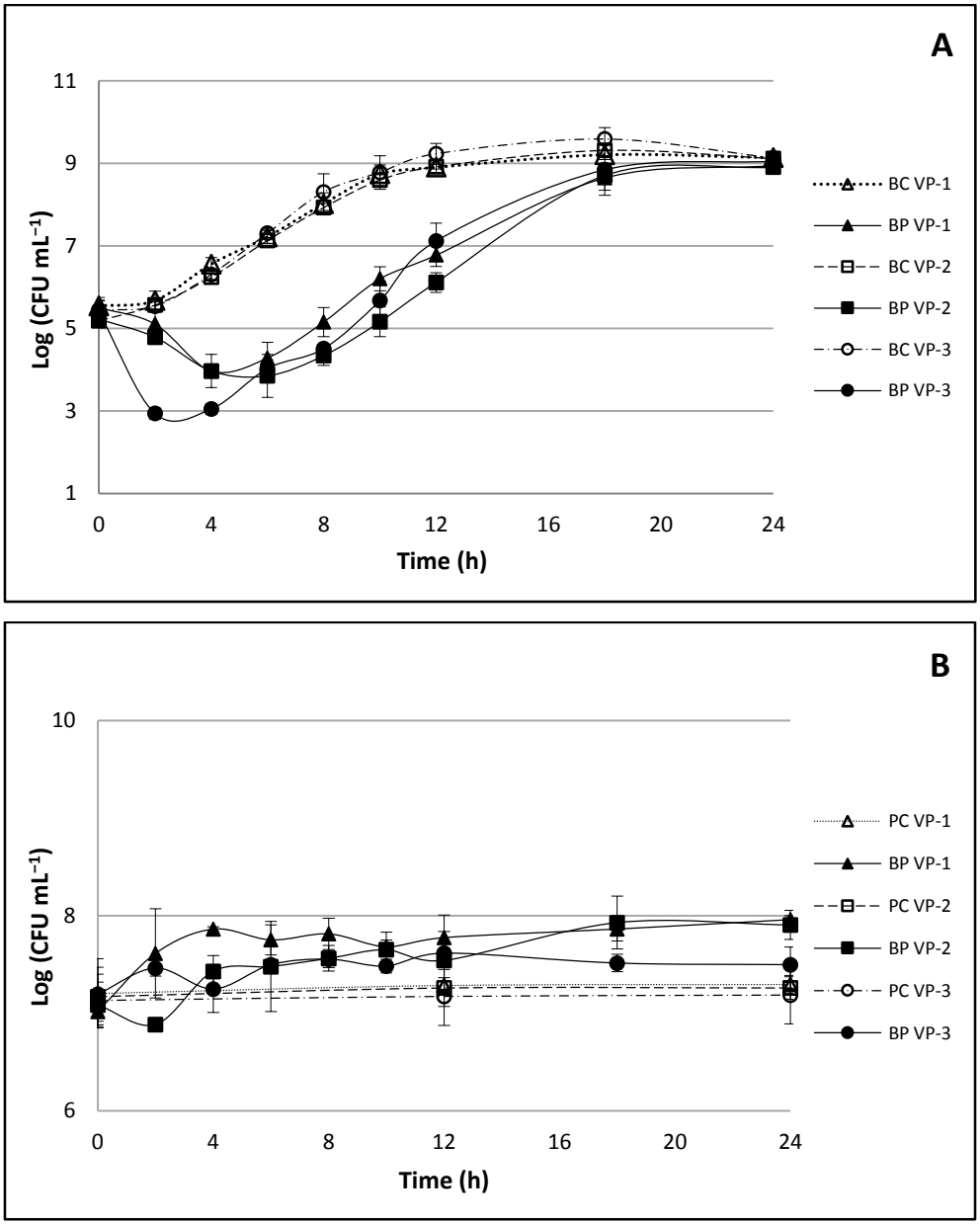

Figure 2.1 - Inactivation of *V. parahaemolyticus* by the three phages (VP-1, VP-2 and VP-3) at a MOI of 100 during the 24 h of the experiments. A. Bacterial concentration: BC – Bacteria control; BP – Bacteria plus phage. B. Phage concentration: PC – Phage control; BP – Bacteria plus phage. Values represent the mean of three experiments; error bars represent the standard deviation.

Phage therapy using phage cocktails

When the phage therapy was performed with the VP-1/VP-2 phage cocktail, the maximum of bacterial inactivation was 4.0 log, achieved after 2 h of incubation, being

statistically different from the values obtained in the phage therapy with the phages alone (VP-1 or VP-2 phage) (ANOVA, $p < 0.05$) (Figure 2.2A).

With the phage cocktail VP-1/VP-3, the maximum of bacterium inactivation was 3.8 log. After 2 h of incubation, bacterial inactivation was significantly different (3.4 log) from the value obtained in the assays of phage therapy with the VP-1 phage alone (0.6 log) (ANOVA, $p < 0.05$). However, when comparing the results of the assays of phage therapy with the VP-3 phage alone with the phage cocktail (VP-1/VP-3), no significant differences were observed after the same period of time (ANOVA, $p > 0.05$). After 4 h of incubation, the rate of bacterial inactivation was 3.7 log, being statistically different from the results of the phage therapy with the VP-1 phage alone (ANOVA, $p < 0.05$) unlike with what was observed for the VP-3 phage (Figure 2.2A).

In the phage therapy assays with the phage cocktail VP-2/VP-3, the maximum rate of bacterium inactivation was 3.6 log after 2 h of incubation, which was significantly different from those obtained in the assays of phage therapy with the VP-2 and VP-3 phages, both alone (ANOVA, $p < 0.05$). After 4 h of incubation, bacterial inactivation was 3.3 log in the experiment with the phage cocktail VP-2/VP-3, and was statistically significant when compared with the therapy with the VP-2 phage alone (ANOVA, $p < 0.05$). However, when compared with the therapy with the VP-3 phage alone, no significant differences were observed for the same period of time (ANOVA, $p > 0.05$) (Figure 2.2A).

When the assays of phage therapy were performed with the three phages altogether, VP-1/VP-2/VP-3 cocktail, the maximum rate of bacterial inactivation was 4.2 log, achieved after 6 h of incubation, being statistically different (ANOVA, $p < 0.05$) from the experiments with the VP-1 phage alone (Figure 2.2A). However, for the same period of time, no significant differences were observed in the assays of therapy with the VP-2 and VP-3 phages alone (ANOVA, $p > 0.05$). Nevertheless, after 2 h of incubation, the rate of phage inactivation was 3.6 log, being statistically different from the values obtained for the phage therapy with the three phages alone (ANOVA, $p < 0.05$). After 4 h of phage therapy, the rate of inactivation with the phage cocktail VP-1/VP-2/VP-3 was 3.5 log, which was not statistically different from the therapy with the VP-3 phage alone (ANOVA, $p > 0.05$), but was significantly different from the therapy with the other two phages alone (VP-1 and VP-2) (ANOVA, $p < 0.05$). After 8 h of incubation, the bacterial inactivation was 3.7 log

in the three phage cocktail experiment, which was statistically different from the values obtained in the phage therapy with the VP-1 phage alone (ANOVA, $p < 0.05$) (Figure 2.2A). Comparing the values of the BC during the 24 h experiment, there were not observed any significant differences among the controls (ANOVA, $p > 0.05$) (Figure 2.2A).

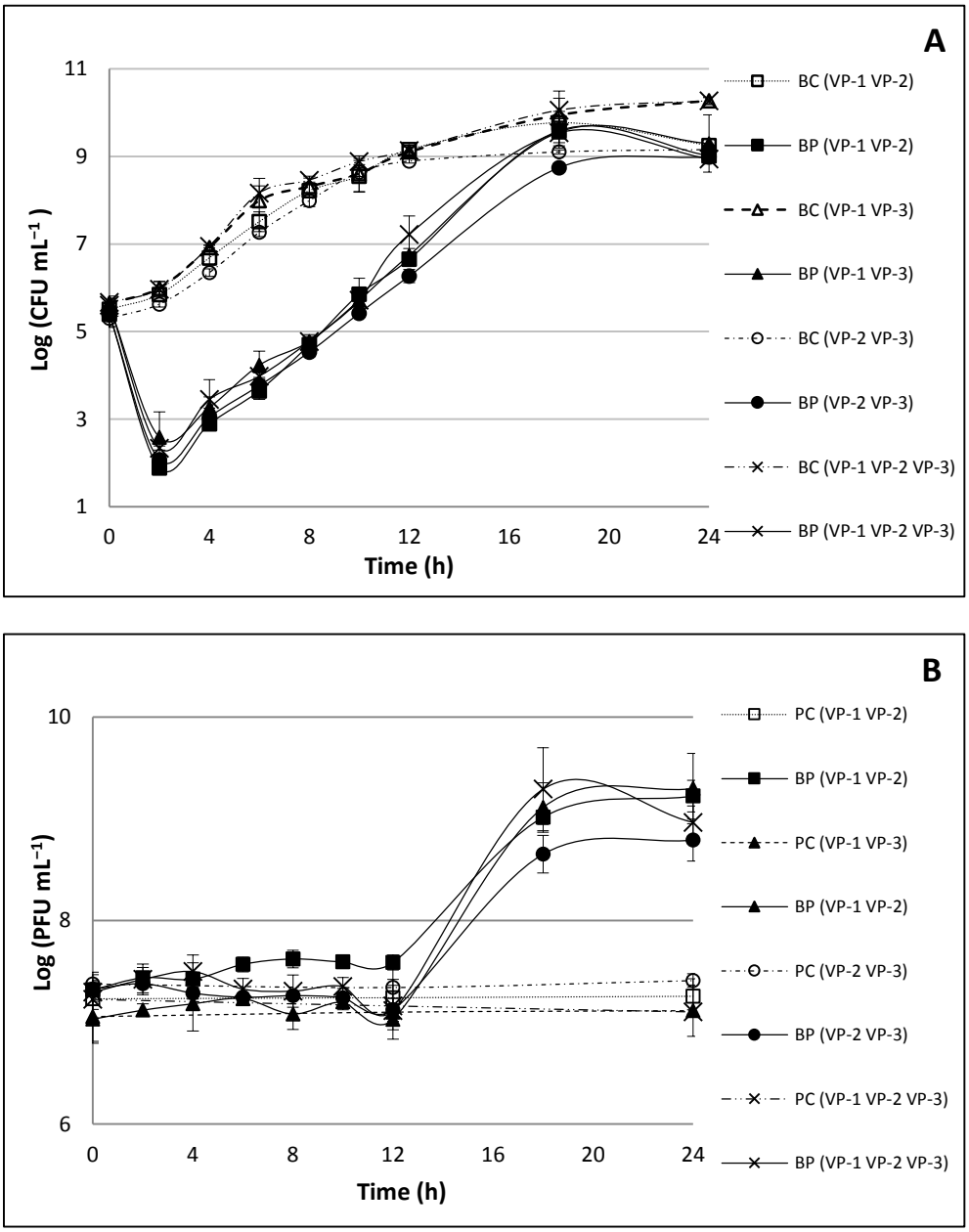

Figure 2.2 - Inactivation of *V. parahaemolyticus* by phage cocktails at a MOI 100 during the 24 h of the experiment. A. bacterial concentration: BC – Bacteria control; BP – Bacteria plus phage. B. Phage concentration: PC – Phage control; BP – Bacteria plus phage. VP-1 – phage VP-1; VP-2 – phage VP-2, VP-3 – phage VP-3. Values represent the mean of three experiments; error bars represent the standard deviation.

No decrease of the phage survival (ANOVA, $p > 0.05$) was observed during the 24 h of the experiments for the VP-1, VP-2 and VP-3 phage controls and for the phage cocktails controls (PC) (Figure 2.2B). The suspensions with the phages alone and with the phage cocktails, when incubated in the presence of the host, presented a significant increase (ANOVA, $p < 0.05$), especially for the phage cocktails (Figure 2.2B). A significant increase of 1.8 log, 2.3 log, 1.5 log and 1.9 log was observed after 24 h of incubation (ANOVA, $p < 0.05$) for the phage cocktails VP-1/VP-2, VP-1/VP-3, VP-2/VP-3 and VP-1/VP-2/VP-3, respectively (Figure 2.2B).

Phage survival in marine water

The phage survival experiments in the aquaculture water collected at different sampling times revealed that all the three phages maintained their viability during long periods in marine water (5 - 7 months). The VP-1 phage remained viable during at least 5 months. VP-1 phage abundance decreased by one order of magnitude during the first day and reached a plateau thereafter, until the 25 th day. Afterwards, the phage titer slightly decreased until the 55 th day and more rapidly up to the 175 th day (Figure 2.3). The VP-2 phage remained viable during at least 7 months. The VP-2 abundance decreased by one order of magnitude in the first day and reached a plateau in the further 12 days. Afterwards, the phage titer slightly decreased until the 35 th day and more rapidly up to the 225 th day (Figure 2.3). The VP-3 phage survival assay showed that this phage remained viable during at least 7 months. The phage abundance decreased by one order of magnitude in the first 20 days, reaching a plateau during the further 55 days. Afterwards, the phage titer slightly decreased until the 85 th day and more rapidly up to the 225 th day (Figure2.3).

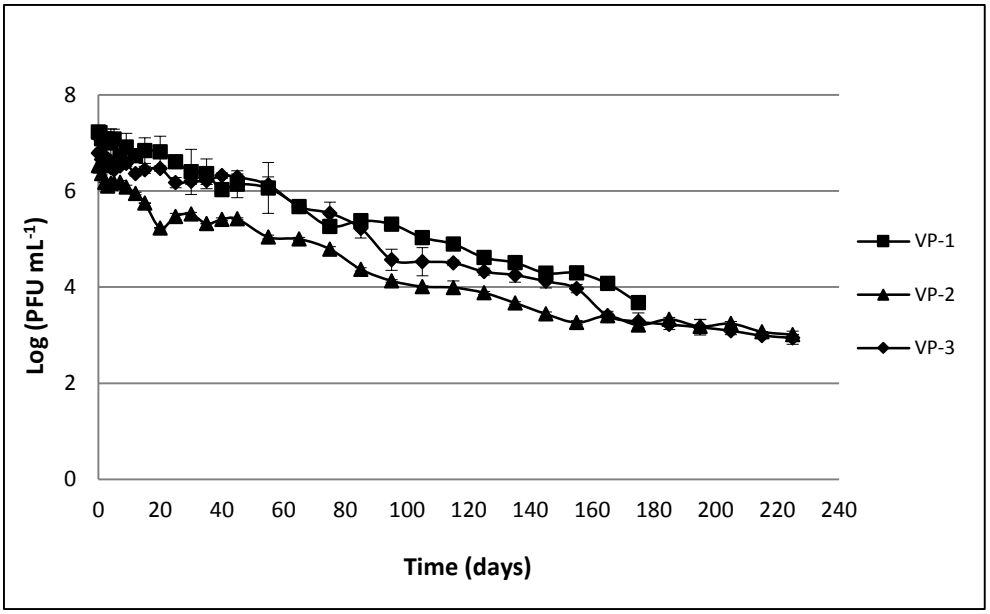

Figure 2.3 - VP-1, VP-2 and VP-3 phage survival in aquaculture marine water. Values represent the mean of three experiments; error bars represent the standard deviation.

Burst size and explosion time

The results of the phage one-step growth experiments revealed that the explosion time of the VP-1 phage occurred at 120 min of incubation. Each infected bacteria produced, in average, 9 phages. When one-step growth experiments were done using VP-2 phage, the explosion time occurred at 90 min and each infected bacteria produced an average of 15 phages. For the VP-3 phage, the explosion time was at 40 min and each infected bacteria produced 42 phages (Figure 2.4).

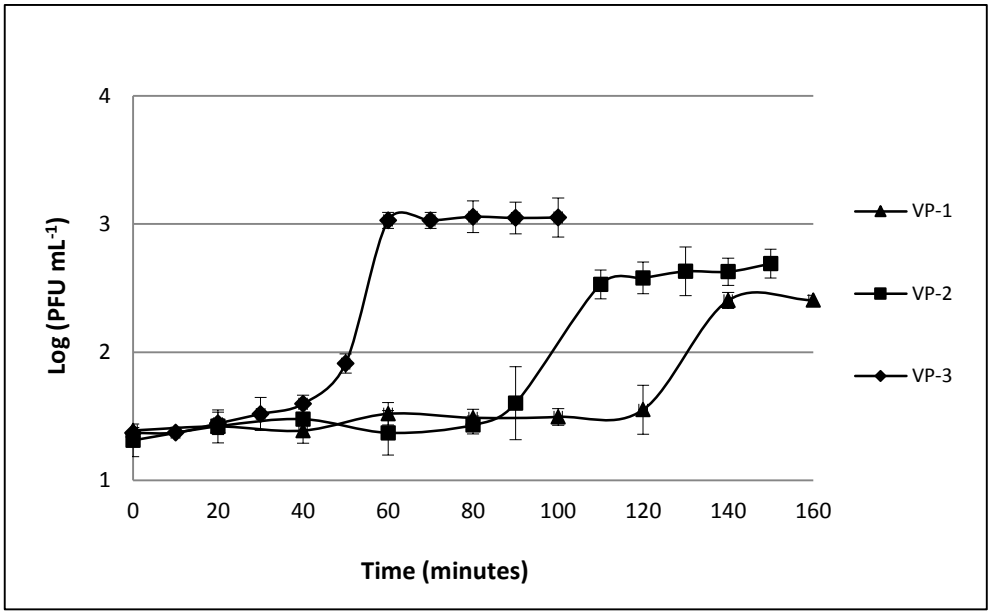

Figure 2.4 - VP-1, VP-2 and VP-3 phage one-step growth experiments in the presence of *V. parahaemolyticus* as host. Values represent the mean of three experiments; error bars represent the standard deviation.

Discussion

Phage therapy is considered to be a viable alternative to antibiotics for the inactivation of bacterial pathogens in aquaculture systems (Nakai, *et al*., 1999; Park, *et al*., 2000, Nakai & Park, 2002, Park & Nakai, 2003, Vinod, *et al*., 2006, Karunasagar, *et al*., 2007; Shivu, *et al*., 2007, Verner-Jeffreys, *et al*., 2007; Higuera, *et al*., 2013; Martínez-Díaz & Hipólito-Morales, 2013). The major challenge of phage therapy is the regular emergence of phage resistant bacteria (Levin & Bull, 2004, Tanji, *et al*., 2005, Merril, *et al*., 2006, Sandeep, 2006, Skurnik & Strauch, 2006, Scott, *et al*., 2007, Nakai, *et al*., 2010, Vieira, *et al*., 2012, Silva, 2013) which imply the ongoing isolation of new phages or variants of phages. However, it has been reported by some authors that the development of phage-resistance can be circumvented by the use of phage cocktails (Crothers-, *et al*., 2010; Filippov, *et al*., 2011, Chan, *et al*., 2013). Although there are few studies about the use of phages to control vibriosis in aquaculture (Vinod, *et al*., 2006; Higuiera, *et al*., 2013), there is not yet any report about the use of phage cocktails to control vibriosis in aquaculture.

Here, we present evidence that phage cocktails can be successfully used to treat *Vibrio* infections. All the phage cocktails tested (two or all the three phages mixed

together) to inactivate *V. parahaemolyticus* were more efficient to control the bacterial growth than the single-phage suspensions. Moreover, with the exception of the VP-3 phage suspension, the bacterial inactivation with the phage cocktails occurred sooner than those obtained using the phages alone. For all of the phage cocktails, a high rate of inactivation was observed after 2 h of treatment, which was only observed for VP-3 phage alone. For the other two phages, the maximum of inactivation was observed only after 6 - 8 h of treatment. However, with the cocktail of these two phages, VP-1/VP-2, the maximum of inactivation was also observed after 2 h of phage addition. The increase in the efficiency of phage therapy (a faster and higher rate of bacterial inactivation) by this phage cocktail, relatively to the use of single-phage suspensions of these two phages, can be due to a delay in the development of phage bacterial resistance. These two phages probably used different bacterial receptors to be adsorb to *V. parahaemolyticus* and, consequently, the time necessary to develop resistance to both phages is higher. However, when the most efficient phage (VP-3), was used in the cocktails, the efficiency of the phage cocktails did not increase significantly comparatively to the use of VP-3 phage alone. This can be explained by the fact that the VP-3 phage may use the same bacterial receptor of the VP-1 and/or VP-2 phages. In fact, when the phage cocktail VP1/VP3 was used, after 2 h of treatment the efficiency of inactivation was not significantly different from that observed for the VP-3 phage alone, but when the phage cocktails VP-2/VP-3 was used the efficiency of inactivation, after the same period of time, was significantly different from that observed for VP-3 phage alone. This suggests the VP-1 and VP-3 phages may use the same receptor to infect *V. parahaemolyticus*. Consequently, in order to delay the development of resistance against the phages, the selection of the phages to be included in a specific cocktail should have into account the type of the bacterial receptor that each phage uses to infect its host. In fact, Filippov, *et al*., (2011) showed that the use of phage cocktails can overcome the problem of bacterial resistance, but only if the phages exploit different receptors. Further studies, including the identification of the bacterial receptors used by the tested phages to infect the bacterium *V. parahaemolyticus*, are necessary to confirm this hypothesis.

The use of several phages in the form of cocktails increases their potential to be used presumptively, that is, prior to identification of the pathogens, and the more phages that are included, greater will be the potential of the cocktail against the pathogenic bacteria.

However, having too many phages in a cocktail could result in a greater impact on non-target bacteria, although in most cases this impact is still less than that expected from typical commercial antibiotics (Chan, *et al*., 2013). Too many phages per formulation can also result in a higher development and manufacturing costs. Moreover, in cocktails containing multiple phages of the same genus with different host ranges, there is a substantial possibility for recombination among such phages to generate new host specificities (Essoh, *et al*., 2013) According to Chan, *et al* (2013), less complex cocktails, for example, two to ten distinct phages, potentially is the best option.

Phage therapy with phage cocktails, as well as with single-phage suspensions, however, did not prevent the bacterial re-growth after treatment. This indicates that although the use of cocktails can retard the development of bacterial resistance, the host can eventually develop resistance against all phages. However, the delay in the development of resistance by the bacteria is an important achievement. Moreover, previous reports suggest that virulent bacteria that are resistant to phage infection could be less fit or could lose their pathogenic properties (Anonymus, 1983, Wagner & Waldor, 2002, Capparelli, *et al*., 2010, Fillippov, *et al*., 2011). Bacterial cell surface components that act as receptors for phage adsorption, can also act as virulent factors that can undertake mutation when the bacteria develop resistance to the phages, being no more pathogenic.

We also showed that the selection of appropriate bacteriophages is a key factor in the success of phage-mediated control of *Vibrio*. Among the criteria that should be required for the selection of the phages to be used in phage therapy are: 1) host range; 2) latent period; 3) burst size, and, 4) survival in the environment. The three dsDNA phages tested in this study (isolated on *V. parahaemolitycus*) 1) infected the same hosts (*V. parahaemolitycus, V. anguillarum* and *A. salmonicida*), 2) presented high periods of survival in marine aquaculture water, however, 3) the VP-3 phage showed higher efficiency for the inactivation of *V. parahaemolitycus* than the VP-1 and VP-2 phages and 4) has a shorter latent period and a higher burst size than the other two phages. These results indicate that the use of phages with high burst sizes and short lytic cycles clearly improve the efficiency of phage therapy. The VP-3 phage, that presents the highest burst size (more than 3 times of those of VP-1 and VP-2 phages) and the shortest lytic cycle (less than an half of those of VP-1 and VP-2 phages), was more efficient to inactivate *V. parahaemolyticus* than VP-1 and VP-2 phages (more 2 log of inactivation, with the maximum of inactivation occurring

4 - 6 hours before than those of VP-1 and VP-2 phages). These results agree with previous reports which indicate that an upsurge in burst size may contribute to larger plaques and higher burst size (Abedon & Culler, 2007).

The three phages infect a similar host range, which can be explained by the fact that all of the phages were isolated using the same strain of *V. parahaemolyticus* as a host. The VP-1, VP-2 and VP-3 phages can inactivate pathogenic bacteria from different families (*V. parahaemolitycus*, *V. anguilla*rum and *A. salmonicida*) with high efficiency. This suggests that these phages can be used not only to treat vibriosis but also furunculose. These results are in agreement with previous studies that showed that some phages may infect more than one related species of bacteria or even genus (Carlton, 1999; Fuhrman, 1999, Thingstad, 2000).

Conclusion

The use of phage cocktails with two or three phages increases the efficiency of phage therapy against *Vibrio,* delaying the development of resistance by the bacterial host, and the use of *Vibrio* phages with high burst sizes and short lytic cycles also increases the efficiency of bacterial inactivation.

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Chapter 3: Effect of lysozyme addition on the activity of phages against *Vibrio parahaemolyticus*

[Submitted to Microbial Biotechnology]

Abstract

Bacterial diseases are one of the major constraints for the development of the aquaculture sector, resulting in huge economic losses. Due to of the alarming level of antibiotic resistance in common pathogenic bacteria and the difficulties with treatment, alternative new methods to control fish pathogenic bacteria are needed. Phage therapy gained increased attention as an alternative to antibiotics in order to control bacterial diseases and prevent the spreading of multiresistant bacteria in aquaculture. However, the effect of lytic enzymes applied during phage therapy has not yet been evaluated. In this study, for the first time, the addition of lysozyme on the activity of three phages of *Vibrio parahaemolyticus* (VP-1, VP-2 and VP-3) to control *Vibrio* infection in aquaculture systems was evaluated. Therefore, different concentrations of lysozyme were tested for the three phages in order to determine the most effective concentrations. The results obtained indicate that the combination of lysozyme and phage showed better activity in comparison to the activity of the phage alone. VP-1 and VP-2 phages in the presence of high lysozyme concentration were more effective in reducing bacterial concentration (reductions of 4.0 log) than the VP-3 phage with lysozyme. However, VP-3 phage (which was the most efficient against *Vibrio*) in the presence of low lysozyme concentration was also effective (reductions of 3.2 log). The addition of external lysozyme can improve the profile of less effective phages during phage therapy but for more effective phages the addition of lysozyme does not produce a so clear effect. Overall, the application of the phage along with lysozyme can be a very useful strategy to eliminate or reduce fish pathogenic bacteria in aquaculture, namely when less effective phages are available.

Keywords: Phage therapy, aquaculture, fish pathogenic bacteria, lysozyme

Introduction

Seafood production in aquaculture provides a good source of high-quality protein and is an important cash income in many parts of the world. It is estimated that the world population obtains at least 20% of its animal protein intake from finfish and shellfish (FAO, 2012). However, microbial disease outbreaks are one of the major constraints for the development of the aquaculture sector resulting in huge economic losses (Hektoen, *et al*., 1995, Oliveira, *et al*., 2012). Microbial diseases are often due to unfavorable conditions like high temperatures, high densities, rapid growth and overfeeding in fish cultivation, infrequent water renewal rate and improper removal of wounded and dead fishes from the farming area (Wahli, *et al*., 2002, Almeida, *et al*., 2009). The typical fish farming diseases are caused by main biological agents like bacteria, viruses, parasites and oomycetes, however, bacterial diseases are a major problem in the expanding aquaculture industry (Alderman, 1996, Wahli, *et al*., 2002, Almeida, *et al*., 2009).

Vibrio species cause vibriosis, a common disease in marine and estuarine fish worldwide, both in natural and commercial production systems, and also occurs in freshwater fishes (Toranzo, *et al*., 1991, Noya, *et al*., 1995, Almeida, *et al*., 2009, Isnansetyo, *et al*., 2009). Vibriosis is caused by bacteria of the genera *Vibrio* (*V. anguillarum*, *V. vulnificus*, *Vibrio parahaemolyticus*, *V. alfinolyticus*) (Toranzo, *et al*., 1991, Hanna, *et al*., 1992, Noya, *et al*., 1995, Sung, *et al*., 1999, Almeida, *et al*., 2009).

V. parahaemolyticus is a halophilic Gram-negative bacterium known to be an important human pathogenic bacterium (Su & Liu, 2007). This bacterium is widely distributed in the marine environments, frequently isolated from a variety of seafood including codfish, sardine, mackerel, flounder, clam, octopus, shrimp, crab, lobster, crawfish, scallop and oyster (Liston, 1990, Wong, *et al*., 2000, Daniels, 2000b, Su & Liu, 2007). It is frequently associated with the development of acute gastroenteritis in human by consumption of raw or undercooked contaminated seafood, particularly shellfish (Kaysner & DePaola Jr, 2000).

Although the administration of antibiotics in aquaculture has been widely used to control vibriosis, this strategy has a serious negative impact on the environment, increasing the problem of bacterial resistance, and leading to their accumulation in the flesh of cultured marine animals (Nonaka, *et al*., 2000, Alcaide, *et al*., 2005, Le, *et al*., 2005,

Sarter, *et al.*, 2007). To reduce the risk of development and dissemination of microbial resistance and to control fish diseases in aquaculture, alternative strategies must be developed in order to improve food quality and safety. Phage therapy in aquaculture seems to be a very promising technique and gained increased attention as a possible alternative to antibiotics, in order to control microbial diseases and to prevent the spreading of multiresistant bacteria in aquaculture (Nakai & Park, 2002, Inal, 2003, Nakai, 2003, Skurnik & Strauch, 2006, Karunasagar, *et al.*, 2007, Shivu, *et al.*, 2007, Verner–Jeffreys, *et al.*, 2007, Higuera, *et al.*, 2013, Martínez-Díaz & Hipólito-Morales, 2013, Silva-Aciares, *et al.*, 2013, Silva *et al.*, 2013).

Although the use of phage lytic enzymes (endolysins) has now been described to reduce the number of an extensive range of bacteria (Tsugita, *et al.*, 1968, Borysowski, *et al.*, 2006, Drulis-Kawa, *et al.*, 2012, Díez-Martínez, *et al.*, 2013), there is no report assessing the effect of phage therapy in the presence of lytic enzymes. Lytic enzymes are encoded by phage genome to facilitate the infection or to destroy the bacterial cell wall. The lytic enzymes used to infect the bacterial cell are components of the virion tail, which are able to locally digest the cell wall from the outside to facilitate the injection of the phage genome into the host cell. These kind of lytic enzymes are widespread in virions, infecting Gram-positive or Gram-negative bacteria (Hogg, 2005). A classic example is the T4 phage lytic enzyme, which is inserted into a baseplate protein of the tail tube, at the tip of the tube (Hermoso, *et al.*, 2007). The lytic endolysins used to weak the cell wall are synthesized in bacterial cells during phage multiplication, by the end of the lytic cycle, they act on the cell wall from inside the cell, facilitating the release of the virions (Fastrez, 1996, Umasuthan, *et al.*, 2013).

Lysins can be classified according to their catalytic activity as lysozymes or muramidases, glucosaminidases, N-acetylmuramoyl-L-alanine-amidases (NAM-amidases), endopeptidases and lytic transglycosylases. Glucosaminidases, lysozymes and lytic transglycosylases act on the sugar moiety (glycosidases), whereas endopeptidases cleave the peptide cross-bridge and NAM-amidases hydrolyze the amide bond connecting the sugar and peptide constituents of peptidoglycan (Hermoso, *et al.*, 2007).

These lytic enzymes, namely the lysozymes, are also produced by other eukaryotes and prokaryotes, being involved in non - specific defence mechanism (Burge, *et al.*, 2007).

As the isolation and purification of these enzymes is easier than those of phages, they can be externally added in order to facilitate the phage penetration during phage therapy.

The aim of the present study was to evaluate the effect of lysozyme from chicken egg white on the activity of three phages of *V. parahaemolyticus* (VP-1, VP-2 and VP-3) in the control of *Vibrio*.

Materials and methods

Bacterial strains and growth conditions

The bacterial strains, *V. parahaemolyticus*, *V. anguillarum*, *A. salmonicida, P. aeruginosa*, *P. fluorescens*, *P. putida*, *P. segetis* and *P. gingeri*, used in this study were previously isolated in our laboratory (Pereira, *et al*., 2011, Louvado, *et al*., 2012). The other bacterial strains, *P. damselae* subsp. *damselae* (ATCC 33539), *E. coli* (ATCC 13706), *V. fischeri* (ATCC 49387), *A. hydrophilla* (ATCC 7966), were purchased from ATCC collection. Fresh plate bacteria cultures were maintained in solid Tryptic Soy Agar medium (TSA; Merck, Darmstadt, Germany) at 4 °C. Before each assay, one isolated colony was aseptically transferred to 10 mL of Tryptic Soy Broth medium (TSB; Merck, Darmstadt, Germany) and was grown overnight at 25 °C. An aliquot of this culture (100 µL) was aseptically transferred to 10 mL of fresh TSB medium and grown overnight at 25 °C to reach an optical density (O.D. 600) of 0.8, corresponding to about 10^9 cells per mL.

Host range determination and efficiency of phage infection

To determine the phage host specificity, the double-layer agar method was used. Spot test was performed with twelve bacteria (*V. parahaemolyticus*, *A. salmonicida*, *V. anguillarum*, *P. damselae* subsp. *damselae*, *E. coli* (ATCC 13706), *V. fischeri* (ATCC 49387), *A. hydrophilla*, *P. aeruginosa*, *P. fluorescens*, *P. putida*, *P. segetis* and *P. gingeri*) to assess the bacterial susceptibility to the bacteriophage (Vieira, *et al.* 2012). The plates were incubated at 37 ºC for 4 - 12 h. The efficiency of plating was determined for the bacteria with positive spot tests (occurrence of a lysis zone) by the double-layer agar method using TSA as culture medium. The efficiency of plating for each host was

calculated by comparison with an efficiency of 100% for the *V. parahaemolyticus* bacterium. For each phage, three independent experiments were done and the results were presented as the average of the three assays.

Phage therapy assays

Phage therapy was performed using three phages (VP-1, VP-2 and VP-3) using the bacterium *V. parahaemolyticus* as host. The assays were performed with single-phage suspensions and with a combination of phage with lysozyme (lysozyme from chicken egg white: Sigma-Aldrich: St. Louis, USA) at different concentrations (0.8, 1.6 µg mL^{-1}, 3, 10 and 20 mg mL^{-1}). Preliminary assays, using the VP-2 phage with low lysozyme concentrations (0.8 and 1.6 µg mL^{-1}) were done. After that, all three phages were tested in the presence of the highest lysozyme concentrations.

Preliminary assays with VP-2 phage in the presence of low lysozyme concentrations

The preliminary assays with VP-2 phage were performed in the presence of two lysozyme concentrations (0.8 and 1.6 µg mL^{-1}). In order to obtain a MOI of 100, 10^5 CFU mL^{-1} of overnight grown *V. parahaemolyticus* culture and a 10^7 PFU mL^{-1} phage suspension were inoculated into 30 mL sterilized glass erlenmeyers with TSB medium. The lysozyme solution was added to two erlenmeyers, which were incubated at 25 ºC without agitation (test samples). Four control samples were also included, the bacterial control without lysozyme (BC), the phage control without lysozyme (PC), the bacterial control with lysozyme (BCL) and the phage control with lysozyme (PCL). The BC was not inoculated with the phage and the PC was inoculated with the phage but without the bacteria. The BCL was not inoculated with the phage but was inoculated with lysozyme, and the PCL was inoculated with the phage and lysozyme but without the bacteria. All controls were incubated exactly as the test samples. Aliquots of test samples and of the bacterial and phage controls were collected after 0, 2, 4, 6, 8, 10, 12, 18, 24 h of incubation for host and phage quantification in the test samples, for host quantification in the bacterial control and for phage quantification in the phage control. The bacterial concentration was determined in duplicate, by pour plating, in TSA medium after an incubation period of 24

h at 25 ºC. The phage titer was determined, in duplicate, by the double agar layer method, after an incubation period of 4 - 12 h at 25 ºC.

Efficiency of VP-1, VP-2 and VP-3 phages in the presence of higher lysozyme concentrations

The final assays were performed for all three phages (VP-1, VP-2 and VP-3) at a MOI of 100. For the VP-3 phage, assays were performed in the presence of three lysozyme concentrations (3, 10 and 20 mg $mL^{-1)}$ and for the other two phages, VP-1 and VP-2, only one lysozyme concentration was tested (10 mg mL^{-1}). In order to obtain a MOI of 100, 10^5 CFU mL^{-1} of the overnight *V. parahaemolyticus* culture and 10^7 PFU mL^{-1} of the phage suspension were inoculated into sterilized glass erlenmeyers with TSB medium (final volume of 30 mL). The lysozyme solution was added to the erlenmeyers, which were incubated at 25 ºC without agitation (test samples). Control samples (BC, BCL, BP and BPL) were also included. Aliquots of test samples and of the bacterial and phage controls were collected after 0, 2, 4, 6, 8, 10, 12, 18, 24 h of incubation for host and phage quantification as described above. Three independent experiments were performed for each condition.

Statistical analysis

Statistical analysis was performed using SPSS (SPSS 20.0 for Windows, SPSS Inc., USA). Normal distributions were checked by the Shapiro-Wilk test and homogeneity of variances was tested by the Levene test. The existence of significant differences among the different phage conditions was assessed by one-way analysis of variance (ANOVA) with the Tukey post-hoc test. For each situation, the significance of the differences was done by comparing the results obtained in the test samples after correction with the results obtained for the correspondent control samples (difference between the control and the test sample) for the different times of each of the three independent assays. A value of $p < 0.05$ was considered to be statistically significant.

Results

Phages host range determination

The VP-1 phage infected *V. anguillarum* and *A. salmonicida*, presenting an efficiency of 83.27 and 64.75%, respectively (Table 3.1). The VP-2 phage infected *V. anguillarum* and *A. salmonicida* presenting an efficiency of 93.39 and 92.03%, respectively (Table 3.1). The VP-3 phage also infected *V. anguillarum* and *A. salmonicida*, presenting an efficiency of 51.21 and 73.78%, respectively (Table 3.1). None of the three phages was effective against *A. hydrophilla*, *P. damselae* subsp. *damselae*, *V. fischeri*, *E. coli*, *P. aeruginosa*, *P. fluorescens*, *P. putida*, *P. gingeri* and *P. segetis*.

Table 3.1- Efficiency of plating (%) of different bacteria.

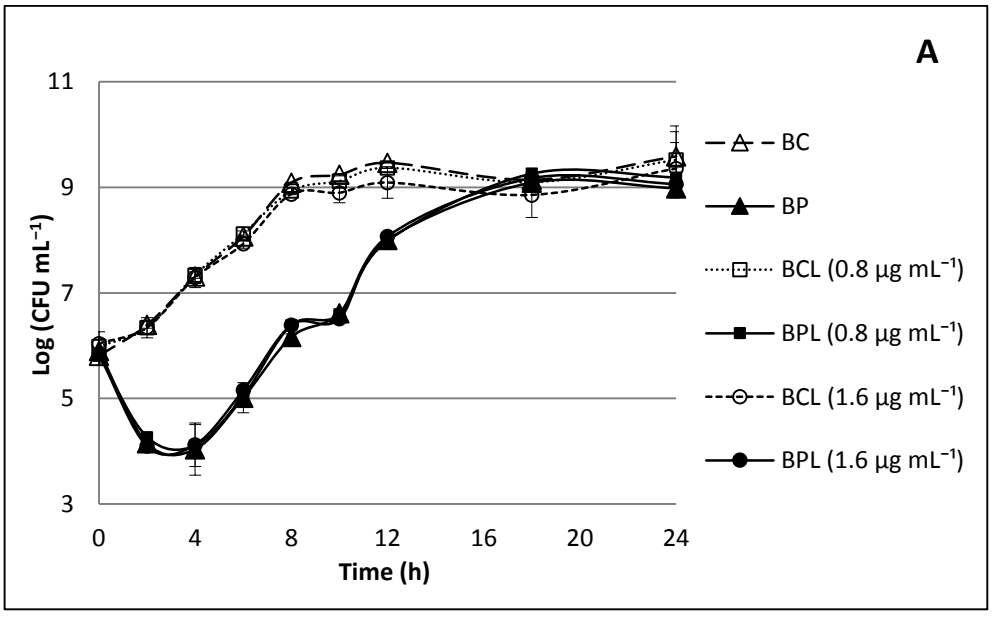
Fish bacteria	**Efficiency of phage plating (%)**		
	VP-1	**VP-2**	**VP-3**
V. parahemolyticus	100	100	100
V. anguillarum	83.27	93.39	51.21
A. salmonicida	64.75	92.03	73.78
A. hydrophilla	0	0	0
V. fischeri	0	0	0
P. damselae subsp. *Damselae*	0	0	0
E. coli	0	0	0
P. aeruginosa	0	0	0
P. fluorescens	0	0	0
P. putida	0	0	0
P. segetis	0	0	0
P. gingeri	0	0	0

Phage therapy experiments

Effect of low concentrations of lysozyme on the efficiency of phage therapy

The maximum bacterial inactivation (ANOVA, $p > 0.05$) with the VP-2 phage was 3.3 log, achieved after 4 h of phage therapy. When lysozyme at the concentrations of 0.8 and 1.6 µg mL^{-1} was added, the maximum rate of bacterial inactivation was 3.2 log for both concentrations, after 4 h of incubation. Comparing the values of the bacterium control without lysozyme with the bacterium control with lysozyme, no significant differences were observed (ANOVA, $p > 0.05$) (Figure 3.1A).

No decrease on the phage concentration was observed (ANOVA, $p > 0.05$) during the 24 h of the experiments for the phage control without lysozyme and for the phage control with lysozyme at 0.8 and 1.6 µg mL^{-1} (Figure 3.1B). When the phage alone and the phage in the presence of lysozyme were incubated in the presence of the host, an increase in the phage concentration (ANOVA, $p < 0.05$) was observed, but significant differences between the two samples were not observed during the 24 h experiment (ANOVA, $p > 0.05$) (Figure 3.1 B).



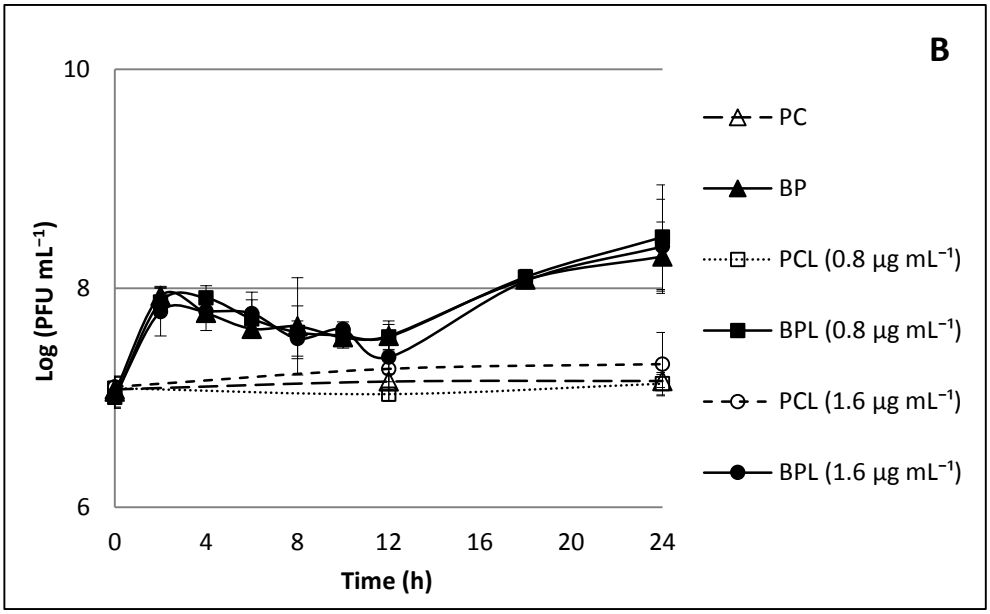


Figure 3.1 - Inactivation of *V. parahaemolyticus* by the VP-2 phage and by the VP-2 phage with lysozyme (0,8 µg mL^{-1} and 1,6 ug mL^{-1}) at a MOI of 100 during the 24 h of the experiment. A. bacterial concentration: BC – Bacteria control; BCL – Bacteria plus lysozyme. B. Phage concentration: PC – Phage control; PCL – Phage control with lysozyme; BP - Bacteria plus phage; BPL – Bacteria plus phage and lysozyme. Values represent the mean of three independent experiments; error bars indicate the standard deviation.

Effect of high lysozyme concentrations on the efficiency of phage therapy

The maximum of bacterial inactivation with VP-1 phage without the addition of lysozyme was 2.9 log, achieved after 6 h of incubation. However, the combination of the phage with lysozyme at 10 mg mL^{-1}, reached a maximum of bacterial inactivation of 4 log after 2 h of incubation, being statistically different from the values obtained with the phage without lysozyme (ANOVA, $p < 0.05$). The difference between the two samples was statistically significant until 10 h of incubation (ANOVA, $p < 0.05$) (Figure 3.2A).

The bacterial concentration in BC and in BCL was similar during the 24 h of incubation (ANOVA, $p > 0.05$) (Figure 3.2A).

Phage survival did not decrease during the 24 h of the experiments, neither for the PC nor for PCL (Figure 3.2B). When the phage alone and the phage with lysozyme were incubated in the presence of the host, a significant increase of 0.7 log and 1.6 log, respectively, was observed after 24 h of incubation, being the difference between both samples statistically significant (ANOVA, $p < 0.05$) (Figure 3.2B).

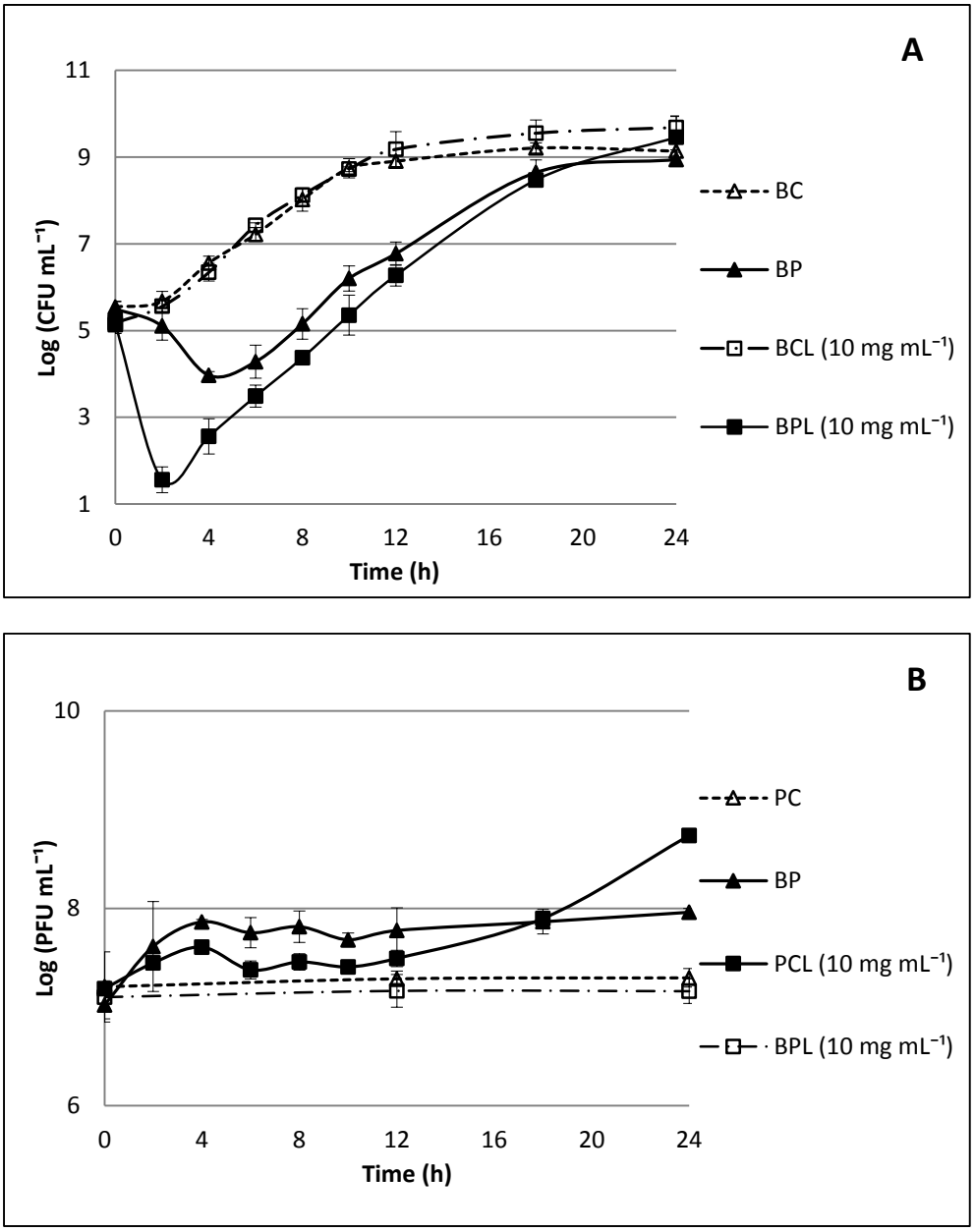


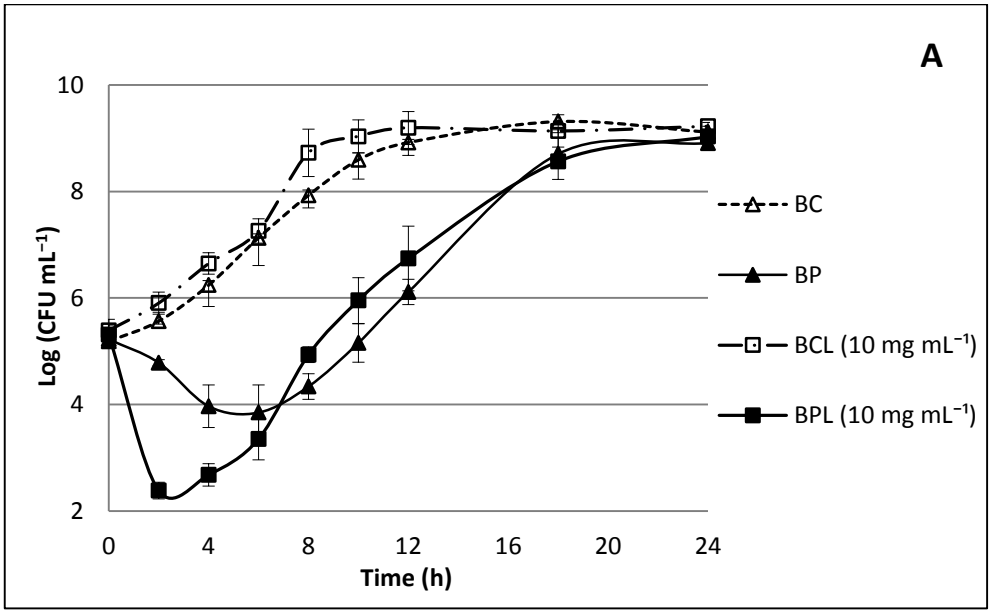
Figure 3.2 - Inactivation of *V. parahaemolyticus* by the VP-1 phage alone and by the VP-1 phage in the presence of lysozyme (10 mg mL^{-1}) at a MOI of 100 during the 24 h of the experiment. A. bacterial concentration: BC – Bacteria control; BCL – Bacteria plus lysozyme. B. Phage concentration: PC – Phage control; PCL – Phage control with lysozyme; BP - Bacteria plus phage; BPL – Bacteria plus phage and lysozyme. Values represent the mean of three independent experiments; error bars indicate the standard deviation.

The maximum of bacterium inactivation with the VP-2 phage without lysozyme was 3.6 log after 8 h of incubation. The assays with the combination of the phage and lysozyme at a concentration of 10 mg mL^{-1} were statistically significant from those without lysozyme, with a maximum of bacterial inactivation of 4 log after 4 h of incubation

(ANOVA, $p < 0.05$). The difference between the two samples was already observed after 2 h of incubation, with reductions on the bacterial concentration of 3.5 log, when lysozyme was added to the samples, and of 0.8 log without the presence of lysozyme (ANOVA, $p < 0.05$). After 12 h of phage therapy the rate of inactivation was still considerably high, but no significant differences were observed between both samples (ANOVA, $p > 0.05$) (Figure 3.3A).

Comparing the values of the BC with the BCL during the 24 h experiment, significant differences were not observed (ANOVA, $p > 0.05$) (Figure 3.3A).

The phage concentration did not decrease during the 24 h, for both the PC and PCL (Figure 3.3B). When the phage alone and the phage in the presence of lysozyme were incubated in the presence of the host, a significant increase of 0.7 log and 1.7 log, respectively, was observed after 24 h, and it was shown that the increase between the both controls was statistically significant (ANOVA, $p < 0.05$) (Figure 3.3B).



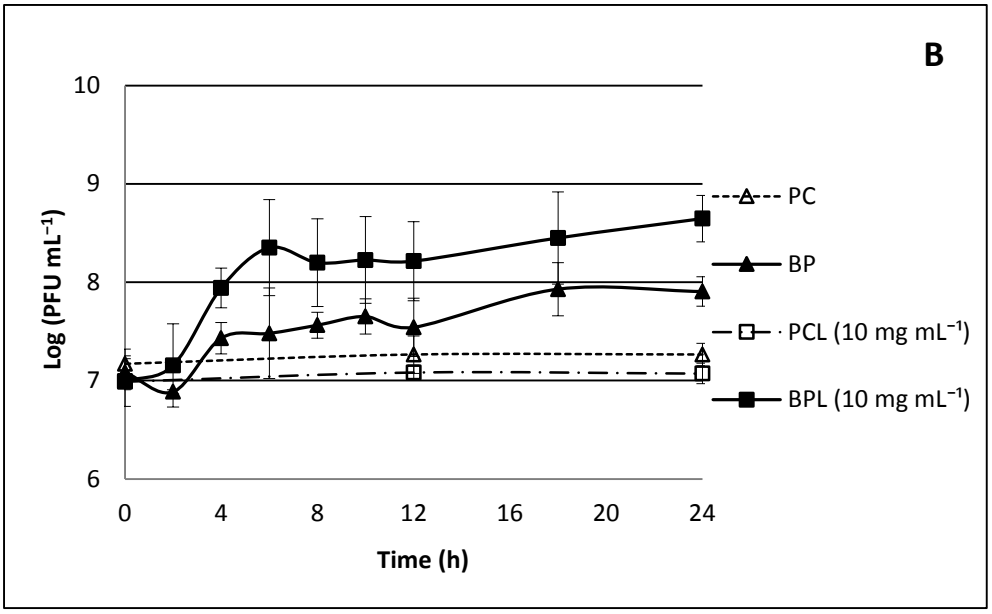
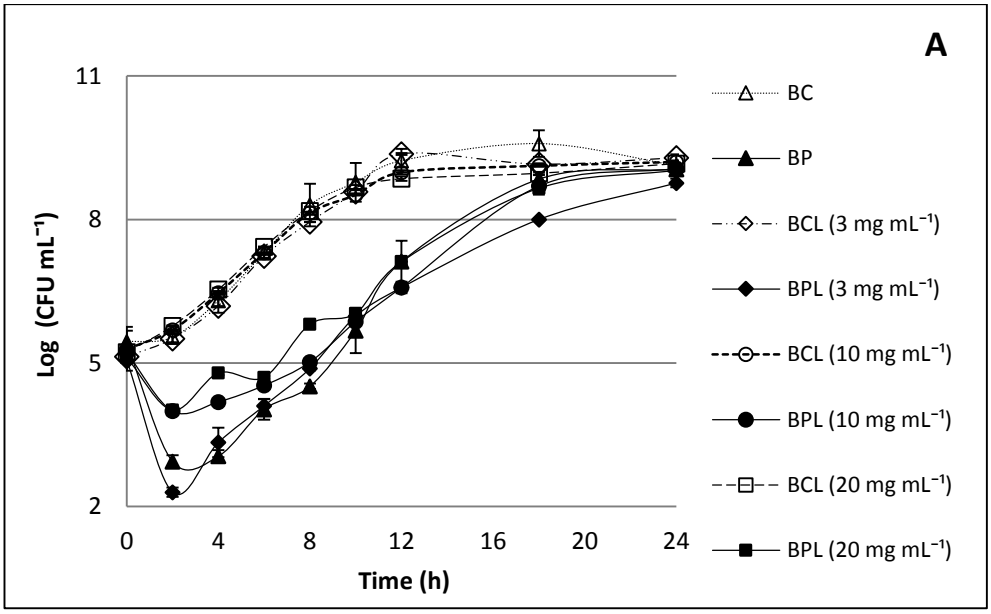


Figure 3.3 - Inactivation of *V. parahaemolyticus* by the VP-2 phage alone and by the VP-2 phage in the presence of lysozyme (10 mg mL^{-1}) at a MOI of 100 during the 24 h of the experiment. A. bacterial concentration: BC – Bacteria control; BCL – Bacteria plus lysozyme. B. Phage concentration: PC – Phage control; PCL – Phage control with lysozyme; BP - Bacteria plus phage; BPL – Bacteria plus phage and lysozyme. Values represent the mean of three independent experiments; error bars indicate the standard deviation.

The maximum of bacterial inactivation by the VP-3 phage was 3.8 log, achieved after 8 h, a value that is not statistically different from the one obtained when the lysozyme (10 mg $mL^{-1)}$ was added to the samples (bacterial reductions of 3.7 log) (ANOVA, $p > 0.05$). For this phage, a higher lysozyme concentration (20 mg mL^{-1}) was tested, but the results were similar to those obtained with 10 mg mL^{-1} (ANOVA, $p > 0.05$) (Figure 3.4A). A lower concentration of lysozyme (3 mg mL^{-1}) was also tested for the VP-3 phage. The bacterial inactivation by the phage in the presence of 3 mg mL^{-1} of lysozyme was only significantly higher than that observed without lysozyme after 2 h of incubation (ANOVA, $p < 0.05$). After 4 h, the efficiency of inactivation was similar for the two samples (ANOVA, $p > 0.05$). However, after 6 h of incubation, the efficiency of phage therapy in the presence of 3 mg mL^{-1} of lysozyme was higher than that observed when lysozyme was used at 10 and 20 mg mL^{-1} (ANOVA, $p < 0.05$) (Figure 3.4A).

Comparing the values of the BC with the values of the BCL during 24 h of incubation, significant differences were not observed (ANOVA, $p > 0.05$) (Figure 3.4A).

Phage concentration did not decrease during the 24 h of the experiments for PC and PCL in the presence of lysozyme at the concentrations of 3, 10 and 20 mg mL^{-1} (Figure 3.4B). When the phage, in the presence of lysozyme (20 mg mL^{-1}) was incubated with its host, a significant increase of 1.0 log was observed after 12 h of incubation. A similar increase was also observed when the phage was incubated in the presence of the bacteria but without lysozyme, however the increase was lower, being significantly different from that observed when lysozyme was added to the samples (ANOVA, $p < 0.05$) (Figure 3.4B).



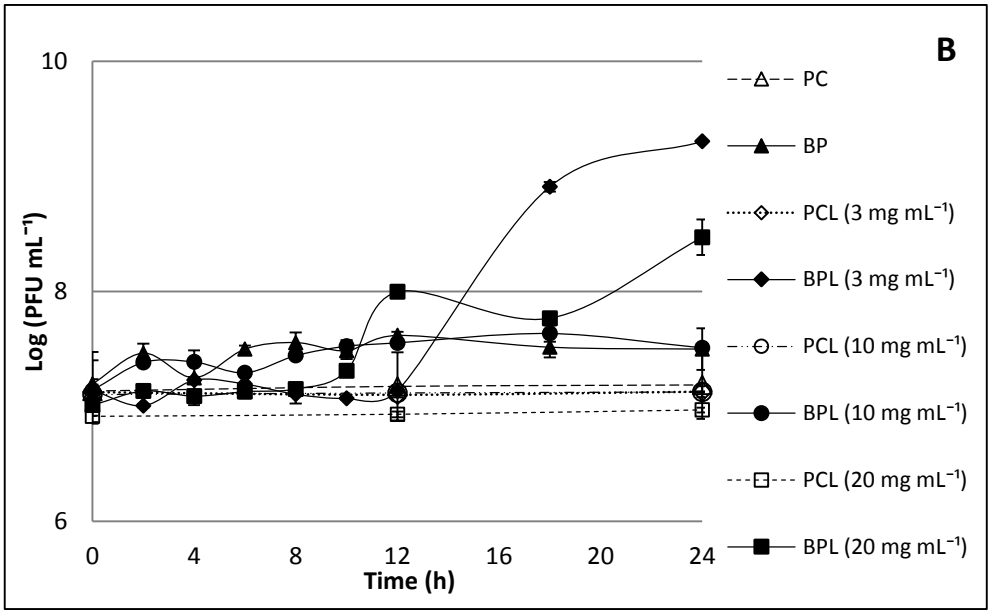


Figure 3.4 - Inactivation of *V. parahaemolyticus* by the VP-3 phage alone and by the VP-3 phage in the presence of lysozyme (3 mg mL^{-1}, 10 mg mL^{-1} and 20 mg mL^{-1}) at a MOI of 100 during the 24 h of the experiment. A. bacterial concentration: BC – Bacteria control; BCL – Bacteria plus lysozyme. B. Phage concentration: PC – Phage control; PCL – Phage control with lysozyme; BP - Bacteria plus phage; BPL – Bacteria plus phage and lysozyme. Values represent the mean of three independent experiments; error bars indicate the standard deviation.

Discussion

The use of phages to inactivate different fish - pathogenic bacteria or to prevent bacterial infections in fish is well documented (Wu, *et al.*, 1981, Stevenson & Airdrie, 1984, Merino, *et al.*, 1990, Nakai, *et al.*, 1999, Munro, *et al.*, 2003, Park & Nakai, 2003, Crothers-Stomps, *et al.*, 2010). However, before phage therapy can be applied, it is necessary to develop effective protocols in order to efficiently inhibit the pathogenic bacteria, avoiding the emergence of resistance to the phages. Although the use of phage lytic enzymes (endolysins) has now been described to reduce the number of an extensive range of bacteria (Tsugita, *et al.*, 1968, Borysowski, *et al*., 2006, Drulis-Kawa, *et al.*, 2012, Díez-Martínez, *et al.*, 2013), there is no report assessing the effect of phage therapy in the presence of lytic enzymes.

In this study, the use of phage therapy in the presence of lysozyme for the control of *Vibrio* in aquaculture was described. The results of this study showed that the addition of lysozyme to the samples positively affected the activity of the phages. However, the

increase on phage efficiency is dependent on the type of phage and, from phage to phage, there was also observed a variation on the optimal lysozyme concentration.

The three phages (VP-1, VP-2 and VP-3), in the presence of lysozyme, showed a higher efficiency to inactivate *V. parahaemolyticus* when compared with the results obtained with the phages alone. Moreover, the lytic effect was not observed when the lysozyme was used alone, which indicates that lysozyme improves the phages entrance into the host cells. The phage lytic enzymes lysis the bacteria, generally in a specie-specific manner (Tsugita, *et al.*, 1968, Borysowski, *et al.*, 2006, Drulis-Kawa, *et al.*, 2012, Díez-Martínez, *et al.*, 2013), but non – phage lytic enzymes are also capable of degrading the peptidoglycan when externally applied (as purified proteins) to the bacterial cell wall, as observed with the egg lysozyme tested in this study. Although the egg lysozyme used in this study is not specie-specific, as the lysozyme alone has no lytic effect, the application of the enzyme with specific *Vibrio* phages turns out the treatment specific.

VP-1 and VP-2 phages, in the presence of lysozyme at 10 mg mL^{-1} (the more effective lysozyme concentration on the activity of the phages), showed a higher efficiency when compared with the results obtained with the phages alone. VP-1 phage, in the presence of lysozyme, achieved the maximum bacterial inactivation (4 log) after 2 h of incubation, which was almost 7 times higher than that obtained with the phage alone (0.6 log). After 10 h of phage therapy, the value obtained in the presence of lysozyme was still significantly higher than that obtained in the assays of phage therapy with the phage alone. VP-2 phage, in the presence of the same lysozyme concentration, exhibited a very similar behaviour when compared with the VP-1 phage. The maximum bacterial inactivation (4 log) was observed after 4 h of incubation and was almost 2 times higher than that obtained with the phage alone (2.3 log). At low lysozyme concentrations the activity of both phages (VP-1 and VP-2) was similar to that obtained with the phages alone. Unlike these two phages, the VP-3 phage only revealed an increase on its activity when lysozyme was added in a lower concentration (3 mg mL^{-1}). The increase of VP-3 phage activity (3.2 log of inactivation) was only significantly different from the values obtained with VP-3 phage alone (2.6 log of bacterial inactivation) after 2 h of treatment (the activity increased by only 1.2 times).

The differences in the activity of the three phages in the presence of lysozyme can be due to differences in infection efficiency of the phages. When the phage therapy was

performed without the addition of lysozyme, the VP-3 phage was the phage presenting the highest rate of inactivation (2.9 log, 3.6 log and 3.8 log for VP-1, VP-2 and VP-3 phages, respectively). In addition, the maximum rate of inactivation for the VP-3 phage occurs soon after 2 h of phage therapy, which does not occur for the VP-1 and VP-2 phages alone (it happens after 6 h and 8 h, respectively). As the VP-3 phage is a very effective phage, the addition of lysozyme showed a low improvement on its activity when compared with the other less effective VP-1 and VP-2 phages. Moreover, the addition of higher lysozyme concentrations (10 and 20 mg mL^{-1}) also did not increase the efficiency of *Vibrio* inactivation by the VP-3 phage.

Such results suggest that the addition of external lysozyme can improve the profile of less effective phages during phage therapy but, for more effective phages, the addition of lysozyme does not so clearly increase the efficiency of phage therapy, and may even cause a decrease in the bacterial inactivation if lysozyme is added at high concentrations. The increase in the efficiency of phage therapy in the presence of lysozyme seems to be due to an increase in the number of phages produced by the bacteria. For the VP-1 and VP-2 phages, the number of phages produced when the lysozyme was added at 10 mg mL^{-1} was significantly higher than those produced when lysozyme was not added. For the VP-3 phage the same was observed for the lysozyme concentration of 3 mg mL^{-1}. Further studies including other phages and bacteria are necessary to confirm these hypotheses. Overall, the application of phage along with lysozyme can be a very useful strategy to eliminate or reduce fish pathogenic bacteria in aquaculture, to a level at which the host immune system is able to take over, namely when less effective phages are available.

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Chapter 4: Conclusions and future perspectives

In this work a serie of experiments to assess the sustainable of phage therapy to control fish pathogenic bacteria, namely the use of phage cocktails and the addition of lytic enzymes during phage therapy was done.

The main conclusions of this work are summarized in the following topics:

- The use of phage cocktails of two or three phages increase the efficiency of phage therapy against V*ibrio*, showing that the phage cocktails can be successfully used to treat V*ibrio* infections. Furthermore, the results indicate that although the use of cocktails did not avoid bacterial regrowth, but retarder the development of bacterial resistance. This retardation is an important reaching, delaying the development of resistance.

- The externally addition of lysozyme, improves the profile of less effective phages during phage therapy (case of VP-1 and VP-2 phage), however for more effective phages the addition of lysozyme does not increase so clearly the efficiency of phage therapy, causing even a decrease in bacterial inactivation if the enzyme is added at high concentrations. Consequently, the application of the phages with lysozyme can be a very useful strategy to eliminate or reduce fish pathogenic bacteria in aquaculture to a level at which the host immune system is able to take over, namely when less effective phages are available.

- The high efficiency of the three phages (isolated on *V. parahaemolyticus*) and their high periods of survival in marine aquaculture water indicate that these phages could to be suitable for use in phage therapy.

- The use of phages with high burst size and short latent period improve clearly the efficiency of phage therapy. In this way, the phage VP-3 that presents a high burst size, and a short lytic cycle was more efficient to inactivate *V. parahaemolyticus* than VP-1 and VP-2 phages.

Regarding, the future work it is necessary to clearer the compression of the mechanisms of phage-resistant in order to improve the efficiency of phage therapy *in vitro* and *in vivo*. It will be also important to test the use of lytic enzymes with cocktails of phages. Moreover, it would be very interesting to evaluate what happens when the one-step-growth curves are determined in the presence of lytic enzymes, in order to evaluate their effect on the burst size and explosion time.