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The prospect of phage therapy in fish hatcheries

PANTELIS KATHARIOS

INSTITUTE OF MARINE BIOLOGY, BIOTECHNOLOGY AND AQUACULTURE, HELLENIC CENTRE FOR MARINE RESEARCH, HERAKLION 71500, CRETE, GREECE

AQUATIC BIOLOGICALS (WWW.AQUATIC-BIOLOGICALS.COM)

EMAIL: KATHARIOS@HCMR.GR

Bacteria and aquaculture

Aquaculture hatcheries are environments of high complexity, where the developing larvae are in a delicate equilibrium with many different organisms, including bacteria, microalgae and live preys, such as rotifers and copepods provided by the fish farmers at the early developmental stages of fish. The role of microbiota at these stages is crucial and only recently have we begun to understand its significance. The establishment of a healthy microbiome in the developing larvae is not only important for their survival but also for their future development and performance during grow out. Environmental and food-borne bacteria shape the gut microbiome of the developing larvae which will be later involved in digestion, immune system development and subsequently growth and survival. Recent studies have indicated vast differences between the various culture systems (e.g. RAS vs flow-through) but also between individual larvae of the same tank (for a review see Vadstein et al 2018). However, it is largely accepted that fast-growing opportunistic bacteria pose the most significant threat in these systems. *Vibrio* is likely the most significant genus of opportunistic bacteria associated with disease outbreaks not only in marine fish but also in other farmed aquatic animals, including crustaceans and bivalves.

Recent advancements in genomic sequencing technology have revealed a very big diversity of *Vibrio* species that were previously misidentified or overlooked because of the resolution limitations of biochemical tests commonly used at the diagnostic labs. In the past, *Vibrio anguillarum* was acknowledged as the most devastating member of the *Vibrio* genus. Now, we know that several other species can be at least equally or even more virulent than *V. anguillarum* such as *V. harveyi* and *V. alginolyticus*. Other species considered as bivalve pathogens are now increasingly implicated in morbidity and mortality of fish larvae like *V. tubiashii* and *V. splendidus*. And as we advance our analytical capabilities it is certain that more species will be added in the pathogenic/opportunistic members of the *Vibrio genus*.

Controlling bacterial populations in the hatchery environment has for long been recognized as critical for sustaining good health and development of fish larvae. Many tools have been or are being used towards this direction. Water treatment through mechanical or UV and ozone filtration, is by far the most commonly employed in aquaculture. In addition to water treatment, many hatcheries "disinfect" live feeds before administration to fish larvae. **Water treatment** and disinfection may seem rational, however this process destabilizes the microbial ecology of the aquaculture systems, providing niche to fast-growing opportunistic bacteria to recolonize the available surfaces (from fish mucosa to physical substrates of the tanks). Another widely studied tool is the probiotics, beneficial bacteria that colonize the fish gut and compete pathogenic microbes. **Probiotics** have good potential, however research towards the sustained and prolonged colonization of the fish gut and towards the use of aquatic vs terrestrial probiotic strains is still needed for improving their efficacy. **Prebiotics** which are non-digestible feed ingredients that selectively promote growth of beneficial bacteria in the gut have also gained attention of the aquaculture industry. Lately, **synbiotics** (combination of pro- and prebiotics) have also been considered as a means of controlling bacteria in the hatchery environment.

All these tools however have one common denominator; they are not specific or targeted. In an ideal situation, an intervention for controlling bacteria should be targeted exclusively to the unwanted or pathogenic bacteria, leaving the beneficial ones unaffected. Phage therapy is such a tool.

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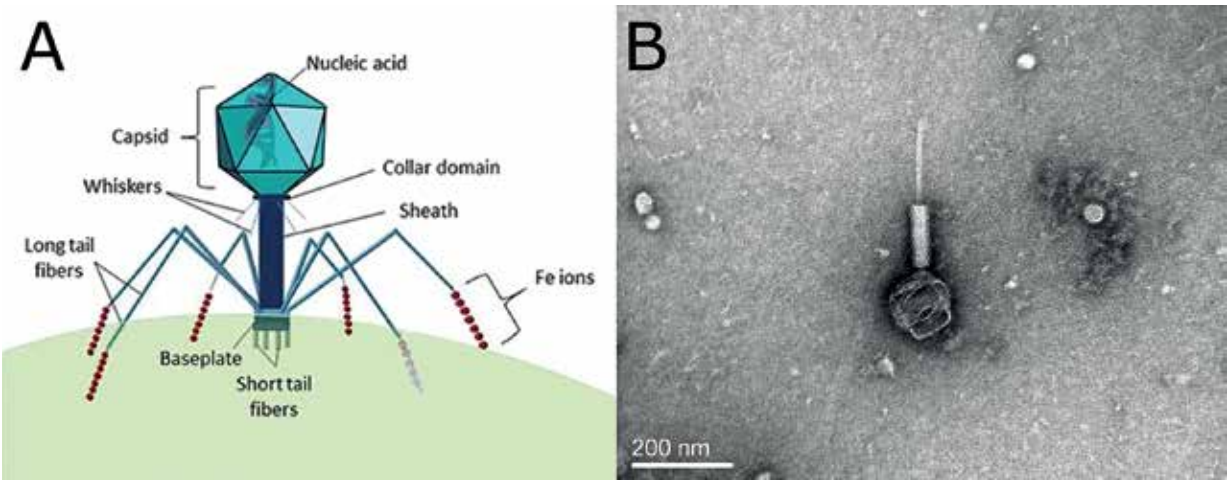


1. A. Schematic representation of the structure of a Myoviridae bacteriophage (by Chelsea Bonnain, Mya Breitbart and Kristen N. Buck licensed under CC BY-SA 4.0). B. Transmission Electron Microscopy image of a Myoviridae bacteriophage showing the contractile tail (courtesy of Dr. Pantelis Katharios)

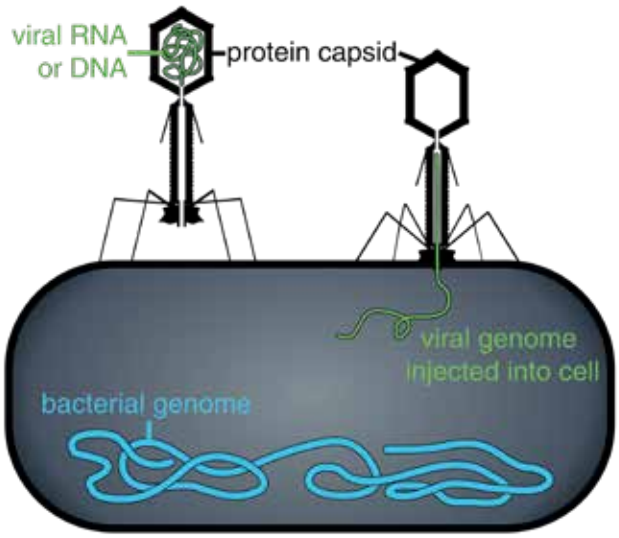
Bacteriophages and phage therapy

Bacteriophages or phages are viruses that exclusively infect bacteria. They are the most abundant life entity[1] in the planet. Their number is astronomical; it has been estimated that there are approximately 10^{31} phages in the biosphere. Phages were discovered more than 100 years ago; initially Ernest Hanbury Hankin, an English microbiologist working in India made the first hypothesis of their existence before the end of the 19th century. In 1915, another English microbiologist, Frederick William Twort published the first scientific paper in the journal Lancet, describing the activity of bacteriophages. But it is Felix d' Herelle, a French microbiologist of the Pasteur Institute who is considered by many the discoverer (and also the name-giver) of bacteriophages. D' Herelle published a paper in 1917 describing bacteriophages as viruses parasitic on bacteria.

Before we examine the potential of phage therapy in aquaculture, we need to discuss some basic notions of phage microbiology.

Bacteriophages are the most efficient "predators" of bacteria in nature. Their ecological significance is huge as they control the number of bacteria in the environment. The structure of phages (**Figure 1**) comprises a proteinaceous capsid that encapsulates their genetic material (DNA or RNA) and in many cases a tail which is attached to the capsid. There are many different morphologies of bacteriophages, tailed and non-tailed, but here we will mostly focus on tailed bacteriophages. Bacteriophages' tails can be long or short, contractile and non-contractile and this character is also used for taxonomical purposes. At the end of the tail there is the baseplate on which there are the tail fibers and the spike. At the distal end of the tail fibers there are receptor binding proteins which interact with specific surface receptors of the bacterial host. Spike proteins display enzymatic activity which is used for the degradation of the lipopolysaccharide layer of bacterial surface to facilitate binding of the phage to the bacterial receptors. Once the phage is irreversibly attached to the bacterial surface it will inject its genetic material inside the cell (**Figure 2**). From this moment on, the phage, as all viruses do, will hijack the bacterial cell machinery for its own purpose which is propagation. There are at least four different types of phages according to their life cycle. The two most well-known and studied are the **lytic or virulent** and the **temperate**. Following its DNA injection to the host cell, a **lytic phage** will start producing its structural proteins and genetic material which will be self-assembled and packaged inside the host cell making up the progeny virions. After the completion of this process, the newly assembled phages will secrete lytic enzymes that will degrade the bacterial cell wall from the inside resulting in a burst that will release them to the external milieu. The number of new virions produced in a single bacterial host cell is called the burst size and can vary significantly between different phages but also between different types of infections. The other type of phage is the **temperate** one. After infection, the DNA of this phage is inserted inside the chromosome of the bacterial hosts. Once the viral DNA is integrated in the bacterial one, the phage (now called **prophage**) becomes "dormant" and replicates along with the bacterium until it is induced by either a DNA damage of the host or following an environmental cue. When the prophage is induced, its DNA is excised from the host's DNA and the phage follows the lytic cycle which was described previously to release the new virions **(Figure 3)**. During the prophage stage (when a temperate phage is integrated in the bacterial host), its genes may become functional genes of that host. Therefore, the infected bacterium which is called a lysogen may carry and express new traits that originally belonged to the phage. The problem arises when such phage genes encode toxins and proteins implicated in antibiotic resistance. It is known that transduction is one of the most commonly observed ways of gene-transfer in bacteria and it is facilitated by the temperate bacteriophages. Such an example is the cholera toxin, which is the main virulence factor of Vibrio cholerae, encoded in a prophage integrated in the chromosome of the bacterium. Likewise, marine pathogenic vibrios like *V. harveyi*, *V. alginolyticus*, *V. vulnificus* and many others carry prophage-encoded toxins which make them more virulent than the non-phage-infected ones. This process of acquiring new properties that may increase the bacterial fitness or more importantly their virulence is called **lysogenic conversion**. And it is exactly this feature that creates the biggest risk in using phages as a therapeutic tool: the accidental transformation of non-virulent bacterial strains to virulent. It is of the greatest importance therefore to select only lytic phages and discard the temperate ones in phage therapy. Moreover, nowadays a more precise selection of appropriate phages is based on genomic analysis. Following whole genome sequencing we can now screen the genomic arsenal

[1] there is an ongoing scientific debate whether viruses are nonliving or living organisms, see: https://www.scientificamerican.com/article/are-viruses-alive-2004/



2. Schematic representation of phage attachment to the bacterial cell and injection of its nucleic acid (licensed under CC)

of phages to discard those who have "suspicious" or unwanted genes. Temperate phages for example carry "signature" genes like integrases required for the successful integration of their DNA into the host's DNA. Modern bioinformatics tools can easily detect this type of genes if the genome of a phage is available.

Phages are usually highly host specific. Sometimes their **host specificity** is down to the strain level. However, there are also phages with a broad host range[2] spanning most commonly different species of the same genus. This feature differentiates phage therapy from all other tools we currently have for controlling bacteria in aquaculture. Phage therapy is a targeted and precise treatment. Host specificity of phages depends on complex molecular interactions between the phage and the bacterium throughout the infection cycle, which is outside the scope of this article. However, one of the most important factors is the type and diversity of receptor binding proteins found on the phage tail, which will be used for the first interaction between phage and bacteria. In Gram-negative bacteria like vibrios, the main target of these phage proteins are the components of their outer membrane like the lipopolysaccarides (LPS) which are major virulence factors, the flagella, and the porins which are receptors used by the bacteria to obtain nutrients from the extracellular milieu.

Phages and bacteria are in a **constant arms race** in the environment. Bacteria are continuously exposed to phage "predation" and in order to survive they must devise strategies to resist phage infection. Bacterial **resistance** against phage infection may develop very fast. Usually, bacteria will sacrifice the receptors to which phages are attached. This is controlled at a molecular level and involves the downregulation of the genes which encode for the proteins of these receptors. Resistance can also be developed through genetic mutations of these proteins that will result in compatibility loss of the phage binding receptors proteins and the receptors of the bacterial cell. But even after the penetration of the bacterial wall, the bacterium has mechanisms that confer resistance against phage infection. The CRISPR-Cas system is actually a bacterial "adaptive immune system" against phages. On the other side, phages also very rapidly adapt and develop counter-resistance measures. Since the ability of bacteria to develop resistance against phage infection is often related to a downregulation of receptors which are being used for nutrient acquisition or by modification of the LPS which is a virulence determinant, in many cases, resistant strains are less fit or less virulent than the wild type[3]. Therefore, the development of resistance has a significant cost for the bacteria. The rapid development of bacterial resistance against phages is one of the main drawbacks in phage therapy. This resistance development is almost certain to happen over a period of time. To overcome this problem, phage therapy should be carefully designed. Combination of different phages termed as "phage cocktails" is the solution. However, the use of the correct ingredients for these cocktails requires expertise and knowledge. In the past, phage cocktails were created with phages displaying different host ranges. Now, we know that a successful phage cocktail should ideally contain phages that use different receptors for the initial attachment to the bacterial host. This is because development of resistance from the bacteria is costly, and changes (like downregulation or mutations) in more than one or two receptors might jeopardize their viability. Lately, phages with very large genomes termed as "jumbo" phages have demonstrated wide host range which is probably related to a wider diversity of receptor binding proteins in their tails. These phages are also very promising ingredients of phage cocktails.

Phage therapy trials in aquaculture

The initial attempt to use phages against fish pathogenic bacteria goes back to the 80s. The first scientific report was from Taiwan and was published in *Fish Pathology* in 1981[4] and regards phage therapy against *Aeromonas hydrophila* infection of loach. Since then, many scientific papers from various countries describe phages with therapeutic potential or use phages as a means of therapy in aquaculture. The majority of the first research trials focused on the use of phages as a method to treat infected fish and the results were variable. In the challenge tests which have been used to assess the efficacy of phage therapy, phages are commonly administered simultaneously with the infectious agent. This results in a significant decrease of the number of the bacteria available for initiating the infection and subsequently in positive results. However, there are very few properly designed studies which would be useful to explore the true efficacy of phages as therapeutics. Ideally, these should use a variety of pathogenic strains of the bacterial target and a phage cocktail that would be administered after the onset of the infection.

The administration of the phages in aquaculture is usually done either directly in the water or in feed. Coating of phages in feed pellets has been proven effective in studies conducted with rainbow trout as the phages could be detected in various organs of the experimental fish after feeding, showing the ability of the phages to survive passage through the fish stomach. Of course, there are several things that should be considered when designing a proper therapeutic scheme. The first is the target bacterial pathogen and the diversity of its strains. Then it is the dose, which is called Multiplicity of Infection (M.O.I.) in phage microbiology and it is the ratio

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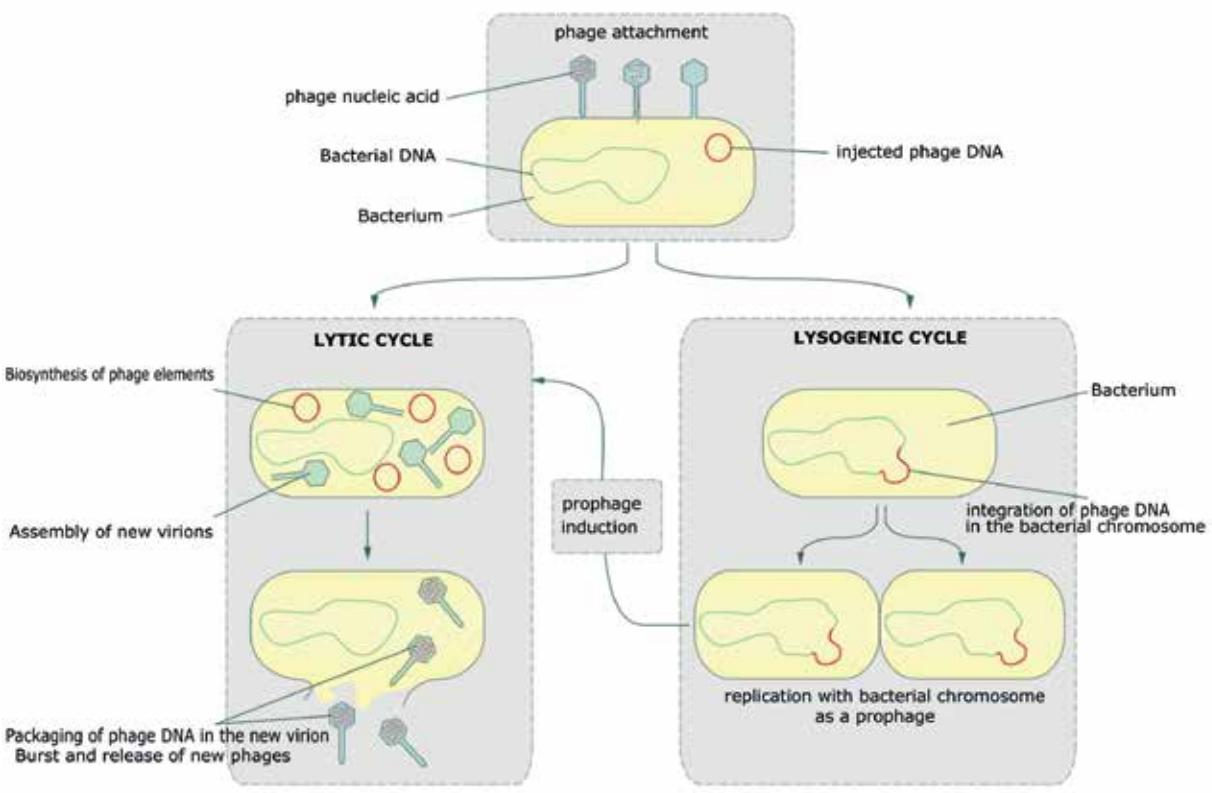
[2] Host range: The taxonomic diversity of hosts a phage can infect

[3] a strain which prevails among individuals in natural conditions, as distinct from an atypical mutant type

[4] Wu, J.L., Lin, H.M., Jan, L., Hsu, Y.L. and CHANG, L.H., 1981. Biological control of fish bacterial pathogen, Aeromonas hydrophila, by bacteriophage AH 1. Fish Pathology, 15(3-4), pp.271-276



3. The lytic and lysogenic life cycle of phages. Lytic or virulent bacteriophages follow exclusively the lytic cycle, whereas temperate bacteriophages follow the lysogenic cycle and are integrated in the bacterial chromosome as prophages. Once induced they will be excised from the bacterial chromosome and will follow the lytic cycle. The diagram is a modification of a diagram licensed under CC.

of phage particles to bacteria. This is determined in the lab during the characterization of the phage. A high M.O.I. suggests that a large number of phages should be used for treatment which of course is directly related to an increased production cost. Another important parameter is the location of the target pathogen (water, mucosa, internal organs, intracellular, etc.).

Phage therapy is very attractive for aquaculture. Bacteriophages are ubiquitous in the aquatic environment. The aqueous nature of the water facilitates their diffusion and increases the likelihood of colliding to the target bacteria. Moreover, phages in the water can pass through the gills and stomach (marine fish drink water) into the blood flow and may reach the internal organs. Since phages are natural inhabitants of the aquatic environment, their medicinal use could be compatible with organic farming. Phage will not leave residues as it is in the case of antibiotics and are completely harmless to fish, humans, and the environment. Phages are viruses which are self-replicating agents and in theory, phage therapy would not require multiple dosing. Moreover, the high host specificity of phages makes them the ideal solution of controlling bacteria in sensitive environments like the fish hatcheries and the RAS. Finally, phages can be used at the early developmental stages of fish where vaccination cannot be applied because the immune system is not mature.

In a recently published research conducted in Finland[5], phages of *Flavobacterium* persisted for 14 days in the tanks of a RAS following single administration. Moreover, the persistence of phages was longer in the biofilters suggesting that biofilm might enhance their survival. This is a very important finding since it suggests that phages can survive the water treatment processes of RAS and can be used prophylactically in those systems to control unwanted pathogenic bacteria in a targeted way.

However, it is the use of phages as a means of controlling bacteria in the hatcheries that has the greatest potential. Even though modern marine hatcheries are areas of increased biosecurity, pathogenic bacteria continue to find their way into the fish rearing tanks causing morbidity, mortality, and inconsistency in the production performance. The administration of live feeds is the vehicle for their entrance. As stated at the beginning of this article, the disinfection of the live feeds will have an impact on the much-needed healthy colonization of the fish gut by beneficial bacteria. A recent use of phages proposed by our group is the use of phages as a "**smart disinfectant**" of live feeds. We have developed and used wide host range phage cocktails that can selectively reduce vibrios in the live feeds. We have shown that following a single administration of vibriophages during the enrichment process of live Artemia for four hours, a reduction of 93% was observed in the vibrio load of the treated group vs the untreated one. We are developing phage-based disinfectants against vibrios of the Harveyi clade like *V. harveyi*, *V. owensii*, *V. alginolyticus* etc. which include serious opportunists commonly found in live feeds. These pathogens are linked to the larval enteritis of gilthead seabream which results in mass losses in many Mediterranean hatcheries. One of the benefits of this method is that the treatment is done in the batch cultures of live feeds, thus significantly reducing the

[5] Almeida, G.M., Mäkelä, K., Laanto, E., Pulkkinen, J., Vielma, J. and Sundberg, L.R., 2019. The fate of bacteriophages in recirculating aquaculture systems (RAS)—towards developing phage therapy for RAS. Antibiotics, 8(4), p.192.https://www.mdpi.com/2079-6382/8/4/192

chances of bacteria to develop resistance against the phages. Such innovative products as the phage "smart disinfectant" are developed by Aquatic Biologicals[6], a spin-off company of the Hellenic Centre for Marine Research which was recently established to develop innovative aquaculture health products.

Similar actions have been documented for salmon hatcheries where bacteriophages have already been used as a biocontrol agent for *Yersinia ruckeri*. This pathogen is responsible for Enteric Red Mouth disease or Yersiniosis. A Norwegian company, ACD Pharmaceuticals has developed and licensed for Norway a commercial phage product that could be used prophylactically to control *Yersinia* in salmon tanks.

Challenges of phage therapy

Although it is more than a century of phage research, phage therapy faces significant challenges before it is widely adopted as a treatment/prevention method at an industrial scale. Culot, Grosset and Gautier, researchers of INRA, France, have recently provided an excellent review of the challenges of phage therapy for commercial aquaculture[7].

The results of phage therapy are still inconsistent. This is mainly due to the improper design of the phage therapy products and application schemes. An extremely important prerequisite for efficacious and safe phage therapy is the thorough characterization of the phages. The elements of phage microbiology which were presented previously in this article need to be studied at the laboratory very carefully. Knowledge of the burst size, the host range, the life cycle and the genetics of any candidate phage will dictate not only how suitable the phage is as a therapeutic agent but also the best way of its application (how much to give, when, etc.)

A very big challenge is to overcome the resistance development from the side of bacteria. As explained in this article, resistance development is the outcome of coevolution of phages and bacteria and it is a natural phenomenon. Phage cocktails will provide the solution, however the formation of potent cocktails is still challenging and requires advanced knowledge and analytical skills.

Mass production of phages is also a very big challenge for the pharma industry. Production of phages in bioreactors is not an easily standardized process. Moreover, in the case where phages are to be used as a therapeutic agent, specific quality standards should be met, like the absence of endotoxins which are released after the lysis of the Gram-negative bacterial hosts. Although this is technically feasible, it significantly increases the production cost.

The biggest challenge, however, is the regulatory barriers found in many countries including the EU and USA. Licensing of phage products as pharmaceuticals is a nightmare. Phages are unconventional pharmaceuticals, a fact that significantly differentiates their licensing process from the accepted norm. More importantly, the ingredients of a phage therapy product should be revised and replaced often to overcome resistance issues. Since every single element of a pharmaceutical product should be extensively tested for safety and efficacy before allowing it as a new component of a licensed product, it is more than evident that licensing of phages will be impossible for the pharma industry. Furthermore, the production of phages as pharmaceuticals at GMP (Good Manufacturing Practices) level is more than challenging and of course extremely costly. There is a strong lobby pushing the regulatory authorities for adjusting the legislation in a way that phage therapy will become an economically feasible and safe option especially in the era of antimicrobial resistance where alternatives are urgently needed.

On the other hand, licensing phage products not as pharmaceuticals but rather as biocontrol agents or water quality enhancers might be a more viable solution for the time being. Companies like ACD Pharmaceuticals have already followed this path and a commercial product based on phages under the trade name CUSTUS is already available in Norway as a biocontrol agent of Yersiniosis in salmon.

Acknowledgements

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Further reading

Readers seeking more information on the topics developed in this article may refer to the selected literature below (open access articles freely accessible for the public):

Vadstein, O., Attramadal, K.J., Bakke, I., Forberg, T., Olsen, Y., Verdegem, M., Giatsis, C., Skjermo, J., Aasen, I.M., Gatesoupe, F.J. and Dierckens, K., 2018. Managing the microbial community of marine fish larvae: a holistic perspective for larviculture. Frontiers in microbiology, 9, p.1820.

Kalatzis, P.G., Castillo, D., Katharios, P. and Middelboe, M., 2018. Bacteriophage interactions with marine pathogenic vibrios: implications for phage therapy. Antibiotics, 7(1), p.15.

Kalatzis, P.G., Bastias, R., Kokkari, C. and Katharios, P., 2016. Isolation and characterization of two lytic bacteriophages, φSt2 and φGrn1; phage therapy application for biological control of Vibrio alginolyticus in aquaculture live feeds. PloS one, 11(3), p.e0151101.

[6] www.aquatic-biologicals.com

[7] Culot, A., Grosset, N. and Gautier, M., 2019. Overcoming the challenges of phage therapy for industrial aquaculture: A review. Aquaculture, 513, p.734423.