Overcoming the challenges of phage therapy for industrial aquaculture: A review

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Review

Overcoming the challenges of phage therapy for industrial aquaculture: A review

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ABSTRACT

Aquaculture is the fastest-growing sector in food industry. Its development is powered by the intensification of the production which increased bacterial disease occurrence and spreading. As aquaculture deeply relies on a massive prophylactic and therapeutic use of antibiotics, it is threatened by the emergence of multi drug resistant bacteria. The stalled development of new antibiotics makes finding new therapeutic solutions a burning issue. Thanks to their specific host range, their ability to treat both the farmed species and the environment, their limited ecological impact and their abundance in the environment, bacteriophages represent a promising sustainable solution to control pathogenic aquaculture bacteria.

In this review we discuss the interest of phage biocontrol for aquaculture and how can bacterial resistance, ecological, pharmacological and production related issues be solved.

1. Introduction

1.1. The rebirth of phage therapy is powered by evolving regulations and new markets

1.1.1. Phages are viruses formerly used to cure diseases

Bacteriophages or phages are viruses that infect and kill bacteria. They were discovered by Twort and d'Herelle at the beginning of the XXth century (Twort, 1915; d'Herelle, 1917). The use of this killing ability against noxious bacteria is called phage therapy. Phages are made of a protein shell measuring between 24 and 200 nm, containing proteins and nucleic acids (Sharma et al., 2017). Those are in general small as their length vary between 17 and 700 kb. The number of genes is also quite small, for example 290 genes were found in the T4 *E. coli* phage (Ackermann, 2003).

Phages can be roughly divided into two groups: temperate phages and lytic phages. These differ by their infection cycle which consists in the integration of phage genetic information into the host's DNA and eventual killing of the host for temperate phages and immediate killing for lytic phages. Temperate phages are generally avoided for phage therapy as they promote horizontal gene transfer and can thereby spread antimicrobial resistance genes or virulence genes, which would be counterproductive (O'Shea and Boyd, 2002; Meaden and Koskella, 2013).

During the beginning of the XXth century, phage therapy showed positive results against plague and cholera in India and phage-based products were commonly available thanks to products like French Bacté-rhino-phage, Bacté-intesti-phage, Bacté-pyo-phage, and Bacté-staphy-phage produced by d'Herelle's company or American Colo-lysate, Ento-lysate, Neiso-lysate, and Staphylo-lysate made by Eli Lilly Company (d'Herelle, 1921; d'Herelle, 1928; Sulakvelidze et al., 2001). This quick development combined to the apparent lack of scientific data on phages incented the US Council on Pharmacy and Chemistry to drive a literature study on the potential and safety of phage therapy which advised to cease therapeutical use of phages (Eaton and Bayne-Jones, 1934a; Eaton and Bayne-Jones, 1934b; Eaton and Bayne-Jones, 1934c). As a result, the Council banished therapeutic phage use and the concomitant discovery of penicillin by Fleming in 1929 marked the beginning of the antibiotic era and tentatively stopped phage therapy research in cold war's western bloc (Fleming, 1929; Krueger and Scribner, 1941a; Krueger and Scribner, 1941b). Between 1960 and 1980, the western bloc's antibiotic to phage therapy publication ratio was close to 10. Meanwhile, the eastern bloc continued to develop phage therapy so that former USSR Eliava Institute in Georgia is now recognized worldwide as a leader in phage therapy research (Elsevier, 2017). Nowadays, as antibiotic resistant bacteria are rising as a global problem, phages and other alternative solutions are reevaluated (Ventola, 2015).

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1.1.2. Western countries are progressively allowing phage industrial use

Today except in former USSR countries, no phage-based product is approved for human use partly due to the lack of suitable regulatory framework and partly due to the lack of data concerning large scale use (Cooper et al., 2016). Nevertheless, phage-based products aiming at improving food safety or agricultural pest management are progressively being granted approval in a few countries around the world. Anti-*Listeria monocytogenes* phage P100, marketed under the product name Listex™, has been Generally Recognized As Safe (GRAS) by US Food and Drug Administration (FDA) and has also been approved as a processing aid for all foods by the US Department of Agriculture (USDA). This product is also approved for use in New Zealand and Australia (Food Standards Australia New Zealand, 2012). Cheil Jedang Corporation pioneered the field, getting one of the first registered phage feed additive product (Biotector®). Finally, other products like EcoShield™ which targets *Escherichia coli* or Agriphage™ (which is used for plant biocontrol) are available on the market (Food and Drug Administration, 2006a; United States Department Of Agriculture Food Safety, 2011; Intralytix, 2018; OmniLytics, 2018).

In the European Union (EU) however, apart from punctual authorizations as in the Netherlands, it is still not officially sanctioned. European Food Safety Authority (EFSA) gave a positive opinion on phage P100 but recommended to validate the efficacy of the product in processing plants and to monitor the evolution of the susceptibility of *L. monocytogenes* to the phage (European Food Safety Authority, 2016; Micreos, 2018). The EU is also funding research projects such as Aquaphage and Enviphage, which aim respectively at building a research network dedicated to the development of phage therapy in aquaculture and to assess the environmental impact of phage therapy industrial use (Aquaphage, 2018; Enviphage Project, 2018). This overall positive trend is backed by a consumer demand for natural and sustainable food-producing processes and entices an increasing number of companies to develop phage-based products.

1.1.3. Consumer demand calls for new antimicrobial products

The rise of the consumption of organic foods and the shift of the food industries toward healthier alimentation reveals a demand for more sustainable food production in developed and developing countries, which translates for instance in the growth of the vegetarian and vegan movements (Luiz Carlos Murauskas, 2016; Marsh and readers, 2016; United States Department Of Agriculture Food Safety, 2017; Gould, 2014). Animal breeding is, according to Food and Agriculture Organization (FAO), among the top three contributors to the most important environmental issues and excessive antibiotics use is one of the reasons of that pollution. It is for instance estimated that half of the US antibiotic consumption is due to breeding (Steinfeld et al., 2006; FAO, 2018a; Food and Drug Administration, 2006b).

The end consumer's demand to reduce antibiotic use meets the food industries' necessity to find new ways to control pathogenic bacteria since antibiotic resistance is a burning issue. Current policies and regulations are discouraging antibiotic use or, as EU's H2020, are encouraging sustainable food production (European Comission, 2017; Medicine, 2017). In this context, phage biocontrol is a promising alternative to antibiotics for the animal breeding industry since they are natural and allow to treat and prevent bacterial infections.

1.2. Aquaculture is a fast-growing industry with major disease management challenges

1.2.1. Current economic importance and growth

Human population has been growing at the fast rate of 75 million people per year between 1971 and 2016 and world population is estimated to reach 9.2 billion in 2050 (Bongaarts, 2009). Aquaculture is the fastest growing food-production industry and seems to be promised to play an important part in feeding the future population. In 2016, it was responsible for 45% of global fish production and this figure is expected to rise to 52% in 2025. Nowadays, aquaculture mainly takes place in Asia where nearly 90% of the volume of fishes for human consumption where produced in 2016 which represents 77 million metric tons. China is the most important country as far as aquaculture is concerned since it produces 62% of world's total production (FAO, 2018b). In 2016, the most produced type of animals were finfishes (68%), mollusks (22%) and crustaceans (10%). To meet projected protein demand, aquaculture production will need to nearly double by midcentury, rising from 80 million tons (Mt) in 2016 to roughly 140 Mt in 2050 (FAO, 2018b; FAO, 2016).

While aquaculture is defined as "the farming of aquatic organisms in inland and coastal areas" which includes many different types of aquaculture: finfish farming, shrimp farming, shellfish farming, algaculture, aquaponics and ornamental fish aquaculture, this review will focus on industrial animal aquaculture only.

1.2.2. The challenge of diseases in aquaculture

As aquaculture progressively grew into an intensive industry, more fishes were concentrated in larger farms which caused an increase in bacterial disease occurrence. Those were treated with antibiotics (Romero et al., 2012). Antibiotics were and are abusively used in some countries insomuch that they can be found after the treatment in sediments and in wild fishes (Defoirdt et al., 2011). Thus released in the environment, these antimicrobials stimulate the emergence of multidrug-resistant bacteria for which new treatments are now needed (Capone et al., 1996; Le and Munekage, 2004; Björklund et al., 1990; Hektoen et al., 1995). For instance, 80% of *Vibrio harveyi* from finfish aquaculture systems in Italy were resistant to amoxicillin, ampicillin and erythromycin (Scarano et al., 2014). Finding new antimicrobials is nowadays a major issue: the last time FDA approved an antibiotic for aquaculture was florfenicol (NADA 141-246) in 2005. The prohibitive costs of development, low return on investment, and very long time (10–15 years) needed to sell a new antibiotic discouraged pharmaceutical companies to release any new one (Food and Drug Administration, 2017; Fowler et al., 2014; Departement of Health, 2013).

Today, the funds required to maintain a healthy aquaculture farm are heavy and are likely to increase if no alternatives are found as antibiotics progressively lose their power and suffer from increasingly strict regulations and social pressure. Finfish diseases are estimated to cost between US $ 1 and 9.6 billion every year which represent between 1 and 10% of the total finfish aquaculture production value. For those and other intensively farmed species like shrimps for which 1990–2005 loss where estimated to $1 billion every year, new and more sustainable treatments are desperately needed (FAO, 2016; Shinn et al., 2015; Flegel et al., 2008).

2. Are bacteriophages a desirable future for aquaculture antimicrobials?

2.1. Phage therapy is especially attractive for aquaculture

2.1.1. Phage therapy is very effective in liquid conditions

The effectiveness of phage therapy partly relies on the ability of phages to reach their host, which is not always granted. In the case of conservation of solid matrixes such as some human foods (hot dogs, smoked salmon…) the efficacy is indeed reduced (European Food Safety Authority, 2016; Ly-Chatain, 2014). Those foods indeed limit the diffusion of phages and therefore limit the effectiveness of the treatment whereas liquid environments allow a deeper impact (Guenther et al., 2009). Other factors such as gut acidity-encountered when using phages to treat mammals for example- can hamper phage use as they can heavily decrease the titer (Watanabe et al., 2007).

In an aquaculture setting however, phages are especially easy to administer since they can access internal organs directly from the water via fish gills. Phage therapy for aquaculture has the benefit to treat both the environment (the water) and the farmed species (Nakai and Park,

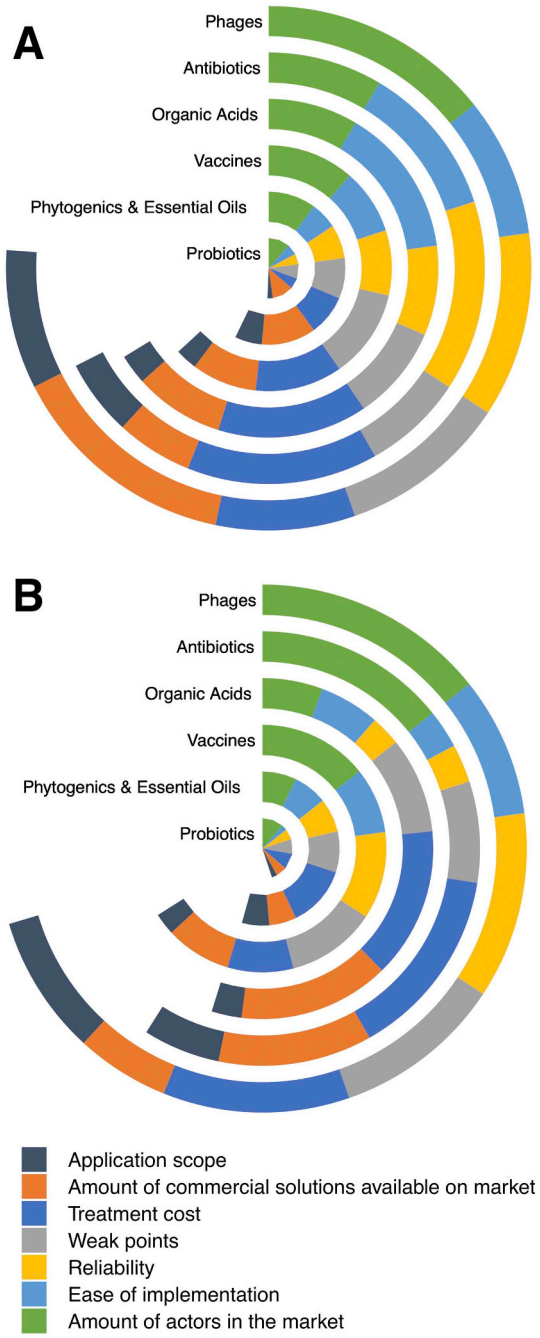

Fig. 1. Comparison of main antimicrobial solutions for aquaculture. Graph A is focused on the European Union market which is mainly represented by salmonids. Graph B is focused on the rest of the world without the EU and is centered on tilapia and shrimp markets. Each solution's score is ranked out of 5 for the 7 criteria: higher is better (see supplemental material). A solution's score is represented by the angle: perfection would be represented by a full circle. When comparing two solutions on a given criteria, readers should thus compare the angles and not the sheer size of the bars. The "weak points" category includes risk of cross contamination, environmental dispersion, environmental side effects, food taste alteration, gene transfer, biochemical interaction with other products, material sourcing difficulty, regulatory hurdles, safety (for both the user and the environment), sensorial disturbance and stressful handling. The "application scope" score is related to the usability of the treatment as a prophylactic or therapeutic treatment. "Reliability" reflects the repeatability of results for each solution. Finally, "ease of implementation" sums the need for diagnosis, development, production, supply chain parameters.

2002; Park et al., 2000).

2.1.2. Phage therapy has numerous advantages over other bacteria control means used in aquaculture

The various existing antimicrobial solutions for aquaculture are summed up in Fig. 1 and ranked according to a commercial point of view: parameters such as treatment effectiveness and market access, treatment cost and regulatory hurdles are taken into account.

As Romero et al. reviewed, antibiotics have a wide range of action and the disruption they cause to fish gut microbiota is likely to impact digestion, nutrition and disease resistance (Romero et al., 2012). In addition to being accused of nephrotoxicity, some of these antimicrobials like oxytetracycline also act as immunosuppressant and thus increase the need for antimicrobials to support the treated fishes' immune system (Grondel et al., 1985; Wishkovsky et al., 1987; Tafalla et al., 2002; Hentschel et al., 2005). On top of the drawbacks of antibiotic use is the fact that they are losing their efficacy which is partly due to the release of these molecules in the environment. It is estimated that 75% of fed aquaculture antibiotics are excreted in the environment and some are even directly added to the water (Burridge et al., 2010). Those massively released antibiotics promote bacterial resistance development, which impedes the prophylactic and therapeutic effects and can eventually be transferred to human pathogen like *Escherichia coli* (Romero et al., 2012). Despite these negative points, antibiotics have a wide range of use and a reliable effect on sensitive strains, which still makes them a noteworthy but unsustainable solution, as Fig. 1 reflects.

Vaccines have been successfully used in aquaculture: they allowed to dramatically reduce antibiotic use in particular for salmon production. However, they are lacking for many farmed species and pathogenic bacteria. Moreover, they cannot be used to protect juvenile fish, which lack of a mature imune system. They also do not always offer a thorough protection and can be tedious to administer when injection is required (Pridgeon, 2012).

Phytogenics are natural plant extracts, the vast majority of which are actually essential oils (Máthé, 2015). Their use seems to be a promising way to control pathogenic bacteria in aquaculture, as they gave positive results in food protection and in *in vitro* studies. On the matter of essential oils, Romero et al. pointed out in 2012 the lack of *in vivo* tests, which are indispensable to allow their use at a large scale and to understand their mechanism of action (Romero et al., 2012). As Sutili et al. discussed, solutions should be found to produce more stable essential oils in order for these products to be market-ready (Sutili et al., 2016). The lack of characterization of a given phytogenic's compounds and of their respective roles is a major hurdle to their use: phytogenics are mainly defined as plant extracts and their compositions is blurry by essence. Sourcing can also be troublesome, as some phytogenic raw materials are hardly available.

Probiotics also represent a plausible alternative to antibiotics. However, their effect are not thoroughly understood either (Romero et al., 2012; Banerjee and Ray, 2017). This problem was highlighted for shrimp farms by Xiong et al. in 2016, as the same probiotics are generally applied throughout the development of the shrimps despite the fact that pathogens vary by the growth stages. As a result, the effect of the probiotics might only appear during one single growth stage thus making it purposeless most of the time (Xiong et al., 2016). Also, it is difficult to combine them with antibacterial solutions especially since virulent and beneficial strains can cohabit in the same species (Austin et al., 1995; Thompson et al., 2010; Noriega-Orozco et al., 2008).

Phage therapy has several advantages over traditional antibiotic therapy: they have a short host range so they only alter the targeted microorganisms without disrupting the whole microbiota, the bacterial resistances seem to be an easier issue since phage are evolving with their hosts in the environment (new ones can be isolated) and phage resistant bacteria tend to have reduced fitness and pathogenicity (León and Bastías, 2015). Bacterial phage resistance seems indeed to have an important fitness cost, as resistant bacteria often show reduced

virulence and growth rate (Santander and Robeson, 2007; Capparelli et al., 2010). This can be explained by the fact that resistance often occurs thanks to phage receptors mutation which can play an important role for the bacteria. Thus, the loss of these receptors can have a dramatic impact on the fitness of the bacteria.

The ubiquitous nature of these viruses is an important advantage of phage therapy. They are considered as the most abundant biological entity on Earth and seem to be present in every ecosystem, from hot springs and deserts to polar areas (Breitbart et al., 2004; Glud and Mathias, 2004; Prigent et al., 2005; Suttle, 2005; Säwström et al., 2008; Lin et al., 2010). Hence it is relatively easy to isolate new phages against resistant or new pathogens. Phage therapy would prove particularly useful on certain aquaculture farmed species like shrimps which lack a specific immune system and can therefore not benefit from vaccines. Shrimp farms can be devastated by AHPND (Acute Hepatopancreatic Necrosis Disease) caused by antibiotic resistant *Vibrio* species from the *Harveyi* and *Orientales* clades and phages could bring a suitable solution to this problem and to other bacterial shrimp diseases such as Pseudomoniasis (Park et al., 2000; Jun et al., 2016).

Among the alternative solutions to antibiotics for aquaculture, phages seem to be part of the most promising which explains the growing interest of private and public research.

2.2. Research is very active but flaws are still to be mitigated before large scale use

2.2.1. Current academic research and private projects

Gon Choudhury et al. reviewed the recent advances in bacteriophage research for aquaculture and summed up the main host/phage associations currently explored (Gon Choudhury et al., 2017). Most of the published articles discuss single phage therapy and report the discovery of new potentially usable phages while the minority addresses cocktails and other associations.

The most studied phages for aquaculture belong to the *Caudovirales* order and to the *Myoviridae*, *Podoviridae* and *Siphoviridae* families. Figs. 2 and 3 were obtained via the analysis of "TITLE-ABS-KEY (phage AND aquaculture)" search on Scopus between 2000 and 2018. Unsurprisingly, Asia is the most active area as far as aquaculture phages are concerned, Europe comes second with nearly a third of the published papers.

Vibriosis is responsible for important economic losses in shrimp farms and farmers lack means of defense since antibiotics are losing their killing potential and vaccines cannot be applied which probably explains the importance of vibriophage research shown on Fig. 2 (Chatterjee and Haldar, 2012). The second most targeted bacterial genus is *Flavobacterium*, namely *F. psychrophilum* which infects

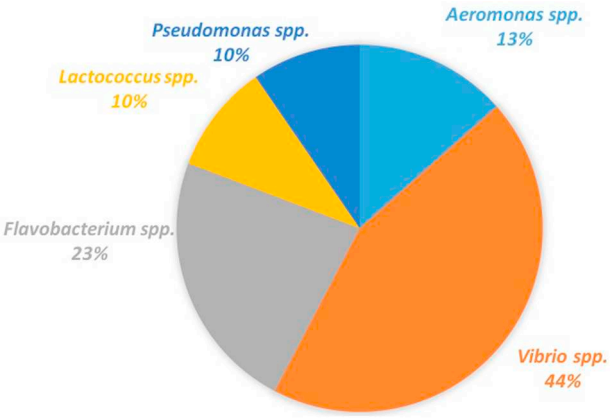

Fig. 2. Most phage therapy research targeted aquaculture bacterial pathogens from 2000 to 2018. *Vibrio* seems to be the most actively targeted genus. Based on Scopus search result analysis (135 articles) (Elsevier, 2017).

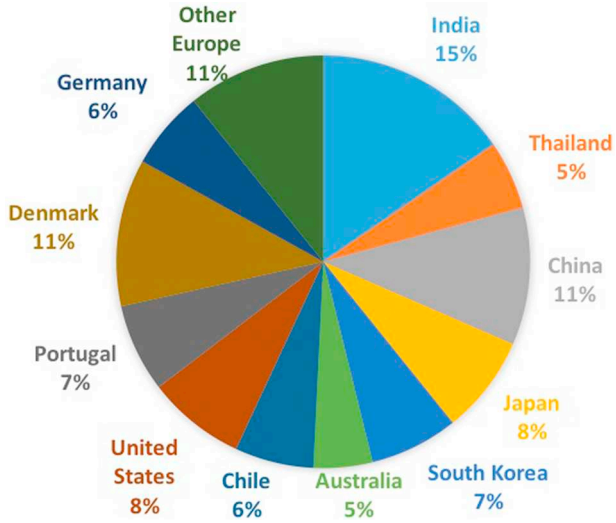

Fig. 3. Distribution of world aquaculture phage academic research from 2000 to 2018. Asia accounts for nearly half of the 135 published articles and Europe represents about a third. Based on Scopus search result analysis (Elsevier, 2017).

salmonids and is not treated by vaccines either and *F. columnare* which infects tilapia, salmonids, eels and catfishes (Long et al., 2014; Declercq et al., 2013). The three least research-targeted species are *Aeromonas spp* which infects salmonids, *Pseudomonas spp* targeting ayu and *Lactococcus spp* mainly infecting trout, tilapia and yellowtail (Fernández-Álvarez et al., 2016; Nishimori et al., 2000; Austin and Austin, 2007).

China, USA and Korea account for the larger numbers of prioritized patents and thus can be considered as leading countries for industrial research for phage used in breeding industries (Fig. 5). These countries are also the most targeted countries for patent extension which indicates that they are perceived as the biggest markets. Although the most active applicants for patents related to phage use for breeding are Korean companies, most of the patenting is done by academic institutions (Fig. 4). This can be explained by the fact that this technology is not mature yet: as phage use in breeding industries develops, the biggest part of patented research will eventually come from private companies. As shown in Fig. 6, aquaculture phage-related patents historically represented a small fraction of the granted patents for the breeding industry. The growing trend seems to show a recent increase in the interest in industrial applications of phage use for aquaculture. A few private companies have publicized their intention to work on phage-based solutions for aquaculture, but few products have currently been commercially released:

- Intralytix is developing a phage cocktail to fight *Vibrio tubiashii* and *Vibrio coralliitycis* infections in oyster aquaculture (Intralytix, Inc., 2016).
- Phage Biotech Ltd works on a phage-based treatment against *Vibrio harveyi* in shrimp hatcheries (Phage Biotech, 2017).
- Proteon pharmaceutical patented in 2017 a product called BAFADOR® which targets *Pseudomonas spp.* and *Aeromonas spp. via* immersion in commercial aquaculture (Grzelak, 2017; Wojtasik et al., 2017).
- ACD Pharma claims to be developing phage-based solutions against several aquaculture pathogens and is full scale testing a product against atlantic salmon yersiniosis (ACD Pharma, 2017).
- Fixed Phage Ltd is developing a technology to bind phages to various surfaces including aquaculture feed pellets (Mattey, 2016).
- Mangalore Biotech Laboratory made commercially available a product called LUMI-NIL MBL for biocontrol of luminous bacteria in shrimp hatcheries (Mangalore Biotech Laboratory, 2019).

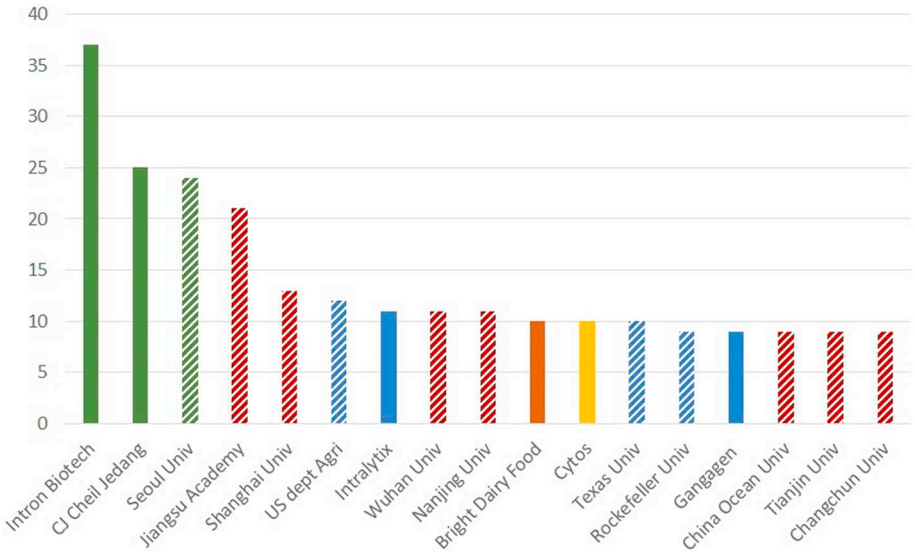

Fig. 4. Main applicants for patents related to phage use for breeding since 2002. Chinese applicants are marked in red, USA are blue, Coreans are green and Swiss are yellow. Hatch-filled bars represent academic applicants and solid bars represent private ones. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.2.2. Phage therapy hurdles for industrial use

Although phage therapy displays several advantages over antibiotic therapy, some of these interesting properties also bring drawbacks for an industrial use: the narrow host range makes the identification of the infectious bacteria mandatory before applying the treatment (Gon Choudhury et al., 2017), resistant bacterial population can nonetheless appear after a phage has been extensively used, phages can promote unwanted gene transfer, a positive public opinion is yet to be made for phages and they are still not authorized in many countries because of the former lack of reliable evidence of such a therapy's safety.

As for mammals, anti-bacteriophage immune response has been identified in fish. As there is little publication on the subject, the impact of this immunomodulation on the success of phage therapy for aquaculture is however still to be evaluated (O'Neill, 1979; Kim et al., 2015). This issue has already been investigated for human phage therapy and a limited effect on treatment efficacy was observed (Żaczek et al., 2016).

Also, phage-based biocontrol solutions need an appropriate formulation depending on the aquatic species, targeted organs, targeted bacteria and allowing a reasonable phage shelf life. The safety of the consumption of phage-treated fishes and the ecological consequences of a massive use of phages must also be assessed before applying what current results present as an ideal substitute for antibiotics at an industrial scale.

3. Releasing phage therapy from its two heavy shackles prior to industrialization: how can we solve the technical problems of bacterial resistance and genetic interactions?

3.1. What are these problems' breadth?

3.1.1. Bacterial resistance and host range

Compared to antibiotics, phages' host ranges are narrow: most studies find them to infect a small fraction of tested strains (Vinod et al., 2006; Kim et al., 2012; Silva et al., 2014a; Kim et al., 2010). Except in a few cases, phages also seem to be genus and even species specific (Ackermann and Dubow, 1987; Bielke et al., 2007; Koskella and Meaden, 2013).

This host specificity is closely related to bacterial phage resistance abilities which can come into play at any step of phage infection (Hyman and Abedon, 2010). A susceptible bacterium can be defined as a suitable host for a complete phage infection cycle. This means that the phage has to evade adsorption resistance, uptake blocks, nucleic acid

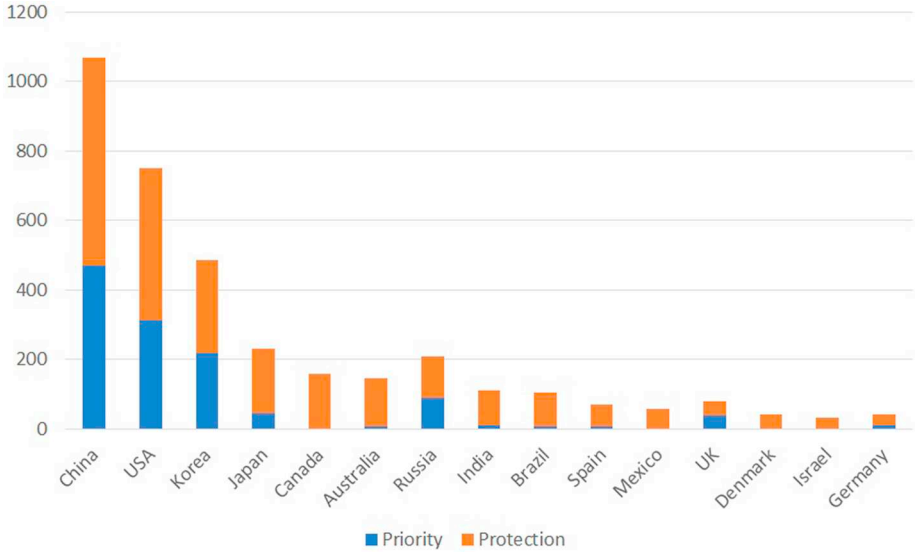

Fig. 5. Distribution of granted and extended patents between countries for breeding since 2002.

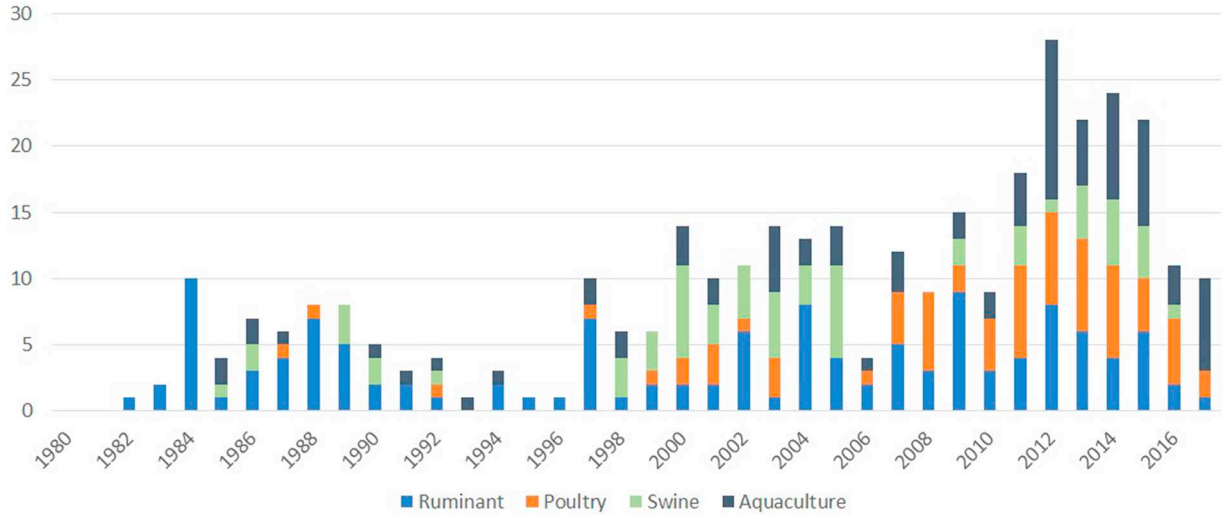

Fig. 6. Number of granted patents from 1980 to 2016 for phage use for aquaculture, poultry, swine and ruminant breeding.

restriction, abortive infection, reduced infection vigor and interference with dissemination in order to reproduce on a bacterial population (Hyman and Abedon, 2010). Phage host ranges are determined by the interactions between phages and potential hosts during all these steps which explains the high specificity of phages for their hosts.

3.1.2. Transfer of critical genes

Phages can spread genes via specialized or generalized transduction thus making them a potential vector for antibiotic resistance genes and virulence genes (O'Shea and Boyd, 2002; Fard et al., 2011). Because of their ability to interact with their host's DNA, phage therapy can be harmful as their use can promote the creation of new pathogenic and/or resistant strains: an extensive study of the genome of every phage involved in phage therapy to search for undesired genes (lysogeny-related and transduction-related genes for example) is needed.

We identified here in part 3 three limitations related to the use of phages as antimicrobials: their host ranges are short, bacterial resistance needs to be fought in order to increase the solution lifetime and they have to be carefully selected to avoid genetic transfers.

3.2. Phage cocktails: union makes strength

3.2.1. Managing host specificity and resistance with phage cocktails

Phage-host interactions are highly specific which implies that bacterial strains causing infections must be identified in order to apply the right phages. To deal with this problem, two solutions can be considered: using specifics phages with an especially large host range or using a phage cocktail containing multiple phages and thus providing a larger host range. A wider host range also allows the use of phages as a prophylactic treatment which reinforces their potential in aquaculture since such treatments are intensively used (Romero et al., 2012). A balance in the host range of a cocktail is however to be found, since a more costly, broader lytic spectrum might promote the unintended selection of resistant bacterial strains or kill untargeted bacteria and thus risking to disturb the beneficial microbiome.

One of the benefits of using multiple phages is to allow a more thorough treatment of an infection since they can target the whole range of pathogenic bacterial strains, with a better bacterial titer reduction and a faster effect thanks to phage synergy kinetics (Schmerer

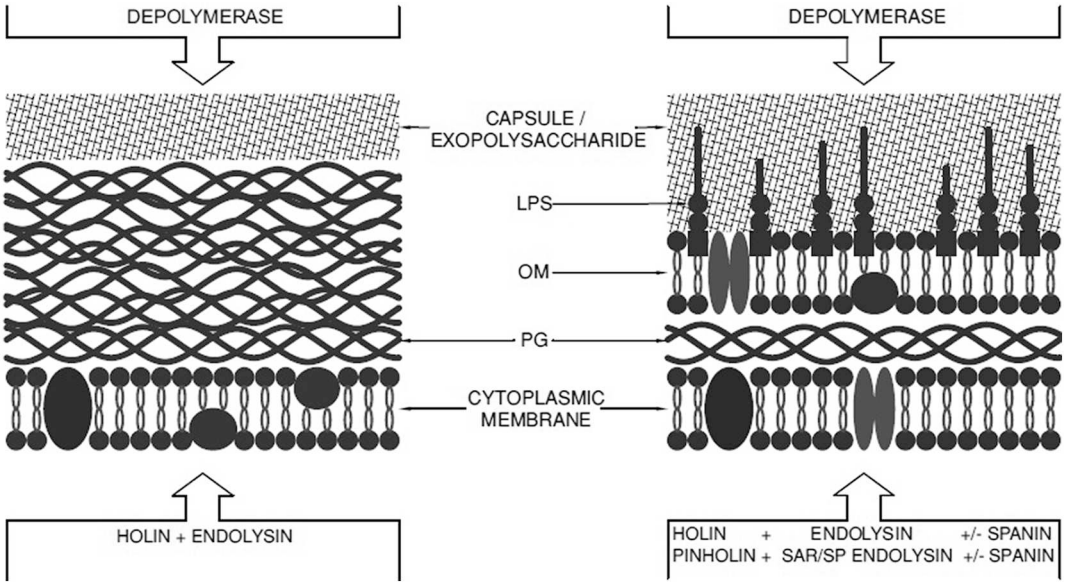

Fig. 7. Location of phage-derived proteins digesting bacterial protective layers. A Gram positive bacteria is represented on the left and a Gram negative bacteria is represented on the right. The main structural differences presented here are the lipopolysaccharides (LPS) displayed in the outer membrane (OM) of Gram negative bacteria and the larger peptidoglycan layer (PG) that can be seen in Gram positive bacteria. Adapted from Drulis-Kawa et al. (2015).

et al., 2014; Mateus et al., 2014). Also, using cocktails composed of phages targeting different receptors of the same bacteria can help slowing down the rate of resistance development (Tanji et al., 2004; Hall et al., 2012; Filippov et al., 2011; Crothers-Stomps et al., 2010). In aquaculture studies, resistance apparition rate to a cocktail typically stands between those of the individual phages and generally varies around 10^{-4} and 10^{-5} CFU/ml (Silva et al., 2014a; Duarte et al., 2018; Pereira et al., 2017; Moreirinha et al., 2018).

Assembling virulent phages into cocktails provides the exciting ability to tailor the treatment for each particular case. A phage library can be built and tested against pathogenic strains isolated from an aquaculture farm to build a more efficient cocktail for that very farm: no excess phage would be included and efficacy could be lab-tested before applying the treatment in the farm. Such tailor-made cocktails are already used in human medicine and would be particularly interesting in industrial treatments where a massive quantity of phages would be needed, since they help reducing the impact on normal microbiota thanks to the absence of "useless phages" (Fevre et al., 2017). This is especially important as impacts of farming practices on the microbiome are raising interest: phage therapy should be compatible with other microbiota management means (Dittmann et al., 2017).

A tailored approach to phage therapy would however be hard to implement for aquaculture since it is time consuming and more expensive: the infected ponds would be devastated before the pathogens could be detected and the matching phages produced and shipped. As discussed in 5.2.1, phage therapy's efficacy is strongly dependent on timing. Spending a few days on isolating and testing the sensitivity of bacterial isolates against a phage library after the apparition of symptoms would thus seriously hamper the success of the treatment. An intermediate approach would consist in studying the periodicity of diseases and producing different location and/or season specialized cocktails throughout the year, as evaluated by Pereira et al. (2011a).

3.2.2. Limits and precautions to phage cocktail construction

Building a phage cocktail should not be done on the sole result that the different phages taken independently are able to kill their target bacteria. For example, phages targeting the same receptor should be avoided as they will compete for it (Mateus et al., 2014). Such a cocktail will at best not display a better killing efficacy than the phages alone despite the higher price of including multiple phages. At worst only a few of them will be able to reproduce enough to maintain the necessary minimal concentration to effectively kill bacteria because of the competition for hosts (Hall et al., 2012; Payne and Jansen, 2003). Using multiple phages targeting the same receptor also promotes bacterial resistance, since a single receptor variation can induce resistance to all those phages (Tanji et al., 2004). Phage interference can also occur in a coinfection scenario, as different phages can compete inside the same host for reproduction. In the case of T4 phages coinfection, lysis inhibition can occur when too big of a cocktail is used (Bourdin et al., 2014; Turner et al., 1999). It seems as a result that caution should be used when increasing a cocktail's size, as it might induce costs that do not translate into positive results. Some authors advise to limit the size of a cocktail to two to ten phages (Chan et al., 2013).

Although lysogenic phages are usually avoided to prevent uncontrolled gene diffusion during phage therapy, lytic phages also present a genetic transfer risk, since nucleic acids are likely to recombine in the event of two closely related phages infecting the same host. Such a reproduction can lead to the "birth" of new phages displaying hardly predictable host range and other properties (Turner et al., 1999). It is thus preferable to avoid including closely related phages, in order to prevent unforeseen damages to the treated farm and the environment.

Finally, even though cocktails are an efficient and bacterial resistance-mitigating way to apply phage therapy, those resistances are still likely to eventually develop and many authors advise to continuously isolate new phages and new hosts to keep up to date with the pathogen-phage evolutionary arms race (Krylov et al., 2016). In order to produce quality cocktails for human phage therapy, some authors recommend to renew at least 1/3 of one's host collection every year (Merabishvili et al., 2018).

3.3. Enhancing phages to avoid the limitation of wild phages

3.3.1. "Natural" ways to engineer phages

Phage breeding is a technique allowing to develop enhanced viruses without directly modifying the phage nucleic acid sequence. Breeding phages is particularly interesting since it is a natural method which therefore evades numerous legal frameworks banishing genetically modified organisms.

S.A.A. Jassim published in 1995 and 2011 two patents describing a so-called breeding method for phages allowing to improve host range and infectivity (Stewart et al., 1995; Jassim et al., 2011). Briefly, this technique consists in selecting the phages yielding the biggest lysis plaques and the fastest infection kinetics among a mixed population of uncharacterized phages amplified from environmental samples (in Jassim's words, this step is called "vertical breeding"). The second step ("horizontal breeding") consists in mixing 20 phages obtained thanks to "vertical breeding" and mixing them with a phage resistant target bacterial strain and a bacterial membrane disrupting chemical agent. After ten days of incubation, the "bred" phages are recovered and are now able to infect the bacteria. Jassim's proposed hypothesis to explain these results is that the disrupted membranes allow previously uninfectious phages to enter the resistant bacteria and complete their infection cycle, while somehow getting the ability to infect their host without the membrane-disrupting agent, maybe by exchanging bacterial receptor recognition genes with those of lysogenic phages already present in the host.

Although claiming the invention of this very promising technique to create improved phages, Jassim and his team did not publish any peer-reviewed article using scientific methods to support his assertions. Furthermore, the two patents detailing the invention fail to provide any scientific proof of the effectiveness of his "breeding" method which effects could be due to many other phenomena than the proposed chemical agent hypothesis. The fact that despite the claimed high efficiency of the breeding method, a 140 bred phage cocktail is used in Jassim's publications demonstrating the effectiveness of his method is also striking and makes it even harder to trust his claims.

This technology has not been independently tested and is advertised in numerous of Jassim's publications (Hibma et al., 1997; Abdulamir et al., 2014; Jassim and Limoges, 2014; Jassim et al., 2016; Jassim and Limoges, 2017). It should be taken with great caution until its inventors provide a scientific proof of their discovery.

To the author's knowledge phage reproduction as discussed in part 3.2.2 has not been exploited yet as a natural way to enhance phages and should be evaluated. A few theoretical studies exist on the subject but there is a lack of papers on recombinaison through coinfection prevalence and on practical applications (Turner, 2003). As described by Merril et al., phages can also be enhanced thanks to directed evolution (Merril et al., 1996).

3.3.2. Genetic engineering

Phage engineering research has produced promising results for phage therapy among which we can cite a modified M13 phage which knocks out an antibiotic resistance mechanism in quinolones-resistant *E. coli*, therefore enhancing the killing effect (Lu and Collins, 2009), a lysogenic phage which was engineered to deliver two antibiotic sensitizing genes to an antibiotic-resistant bacterium (Edgar et al., 2012) and finally a phage that was engineered to carry a lethal gene to its host (Westwater et al., 2003).

Other modifications of phages aimed at solving the main issues associated with their use: namely host range, gene transfer and endotoxin release upon massive bacterial lysis. The end of most of phage's lytic cycle features the expression of holins and endolysins. Holins are

responsible for creating a hole in the plasma membrane thereby killing the host by clearing away the membrane potential. Endolysins are responsible for dismantling the peptidoglycan. The destruction of the peptidoglycan leads to the destruction of the cell membrane and the release of toxic membrane components (endotoxins) which might in turn trigger a septic shock if the bacteriophage infection takes place inside the fish orgnism (Hagens and Blasi, 2003; Slopek et al., 1983; Lepper et al., 2002). Some research teams managed to solve this issue by making lethal but non lytic phages, expressing only holins or destroying its host DNA (Hagens and Blasi, 2003). In the same spirit, Matsuda et al. engineered a lysis-deficient phage able to bring the survival of infected mice from 50% to 80% compared to the wild-type, with even lower endotoxin levels (Matsuda et al., 2005). Mahichi et al. modified a T2 phage's tail with IP008 phage's tail proteins thus granting it with the latter's broader host range while keeping the lytic activity of the T2 phage (Mahichi et al., 2009). Engineered phages were also made that targeted an even tighter range of hosts or that lacked the ability to replicate thus dramatically reducing their potential impact on the environment. This loss of ability to replicate comes at the cost of the loss of ability to replicate specifically at the site of infection which is especially interesting since it compensates for the low diffusion rate in living tissues of phages compared to other therapeutic molecules (Hagens et al., 2004; Citorik et al., 2014; Bikard et al., 2014; Doss et al., 2017; Dubin et al., 1970).

The difficulty for phages to target obligate intracellular bacterial pathogens (such as *Chlamydia trachomatis*) is often cited as a limitation to phage therapy since the prokaryotic cell is shielded from the phage by the eukaryote host. Despite this, phages are able to cure intracellular bacterial parasite-mediated diseases (Kiknadze et al., 1986). This most likely happens thanks to the entrance of virions inside the eukaryote host cell, attached to the parasitic bacteria and only infecting the prokaryotic host after the completion of this step (Hsia et al., 2000). To improve its therapeutic potential against *C. trachomatis* infections, Bhattarai et al. engineered a phage by improving its ability to be endocytosed by the eukaryote cell (Bhattarai et al., 2012). This technology could be used to treat fish diseases caused by intracellular bacterial pathogens, like *Piscirickettsia salmonis* which infects salmon (Fryer and Hedrick, 2003).

Modified phages do not seem to have shown their full potential yet. In the future, chimeric phages built out of the best component of other phages could be created. As Pires et al. noted, such phages could also fit better in the current regulatory framework in which cocktails are hard to register since each phage must be individually approved (Pires et al., 2016). A single very efficient engineered phage may indeed be easier to register than a cocktail composed of many phages. The ability to create extremely efficient, non-replicative phages is also a promising prospect, as it would prevent any uncontrolled diffusion of such an artificial organism in the environment (and its hardly predictable consequences) which is one of the main issues with genetically modified organisms (GMO).

Although these engineered phages have a promising potential, most of them are unlikely to be actually commercialized even in a distant future. First, using lysogenic phages or modifying the targeted bacteria's genome *in situ* are hazardous approaches because they may lead to an uncontrolled environmental spread of the inserted genes. Second, the techniques used in the cited articles are costly and not suitable for the modification of a high number of phages. Third, natural phages use is already a cumbersome endeavor because of nowadays regulations which makes the global use of GMO phages very unlikely in a near future. Finally, GMOs suffer from a really bad public reputation which is yet another hurdle in the path of an industrial use of engineered phages.

3.4. Phage therapy without phages

The many advantages of traditional bacteriophage therapy using whole virions come along with a few drawbacks: phages are big compared to usual therapeutic molecules and hence circulate slowly. They also carry genetic information which might spread in the treated environment. One of the solutions proposed to solve these issues consists in using only phage proteins instead of whole virions. As reviewed by Drulis-Kawa et al., multiple phage proteins could be used to kill bacteria; they belong to two main groups: Virion-Associated Phage Hydrolases (VAPH) and cell envelope digesting proteins which are mainly endolysins (Drulis-Kawa et al., 2012; Drulis-Kawa et al., 2015). VAPH are depolymerases that allow virions to get to their host prior to infection by digesting the extracellular polymeric substances layers protecting the bacteria, whereas endolysins are expressed at the end of the lytic cycle and are responsible for host lysis. All of these molecules are specialized in degrading some of the bacterial protective layers (Fig. 7).

The most interesting features of phage-derived depolymerases as therapeutic agents are maybe their substrate specificity (which allow to target a restricted array of bacterial species), their better stability compared to virions (which grants a longer shelf life), the assumed lack of bacterial resistance and their protein nature (which makes these solutions safer and less tedious regulation-wise than whole virions) (Sutherland, 1999; Sutherland, 1995; Ackermann et al., 2004; Becker et al., 2008; Loeffler et al., 2001; Schuch et al., 2002). The applications vary from a phage-derived protein to another: endolysins are generally ineffective against Gram - bacteria which membranes are shielded by exopolysaccharides but VAPH can go through this layer which makes them a potential treatment against these bacteria and against undesired biofilms. Rodríguez-Rubio et al. wrote a detailed review of these applications, some of which are already on the market for human health and could maybe be used for animal health and feed markets (Rodríguez-Rubio et al., 2015).

While little results have been published in the aquaculture field, we can still note the discovery of an endolysin targeting AHPND-associated *Vibrio parahaemolyticus* (Zermeño-Cervantes et al., 2018). Although endolysins and VAPH are under the spotlight as far as phage-derived proteins are concerned, they are obviously not the only phage proteins involved in host lysis and other new antimicrobials could be yielded by phages (Drulis-Kawa et al., 2012).

3.5. Combining phage therapy with other solutions

3.5.1. Mixing phage proteins with other antimicrobials

As discussed by Schmelcher et al., the already attractive properties of phage-derived proteins can be augmented thanks to genetic engineering (Schmelcher et al., 2012). Although, supplementing them with other molecules could be a very promising strategy. Using multiple antimicrobial agents at once is especially interesting when the total effect exceeds the sum of the individual effects, in other words when the combined effect is synergistic. Such an effect has been observed with phage-derived proteins associated with nisins and could also be applied to whole virions (Becker et al., 2008; García et al., 2010).

3.5.2. Giving a second chance to antibiotics with Phage-Antibiotic Synergy

In 2007, Comeau et al. observed extended phage-induced lysis plaques around β-lactam antibiotic discs and called the phenomenon Phage-Antibiotic Synergy (PAS) (Comeau et al., 2007). The previous discovery of this phenomenon occurred in 1945 before the antibiotic crisis and had a limited impact at the time (Himmelweit, 1945). What makes this phenomenon especially interesting is the fact that the enhanced killing effect of phages is observed at sub Minimal Inhibitory Concentration of the antibiotic (MIC). This effect was observed by numerous other researchers on lytic and lysogenic phages with different quinolone, aminoglycoside and β-lactam antibiotics (Kamal and Dennis, 2015; Ryan et al., 2012; Torres-Barceló et al., 2014; Sagar et al., 2017; Chaudhry et al., 2017). In a few studies, best efficacy was obtained when the antibiotic was added a few hours after the phage (Torres-

Barceló et al., 2014; Lopes et al., 2018).

The improved killing effect seems to be caused by an increase in host burst size (Comeau et al., 2007; Kamal and Dennis, 2015). This increased number of virions is also produced faster since the eclipse period -which spans from the beginning of the host infection to the moment where the first virion is completed- is reduced, as Ryan et al. demonstrated (Ryan et al., 2012). PAS has been first observed with low antibiotic concentrations and increases as the concentration rises. It can however, be inhibited by even higher concentrations (Chaudhry et al., 2017; Knezevic et al., 2013). This inhibition was observed with an aminoglycoside antibiotic and with a quinolone antibiotic which respectively disrupt the synthesis of proteins and disrupt the synthesis of RNA: the negative effect could be due to the inhibition of the synthesis of phage components which are proteins and nucleic acids. The replication of the phage is thus prevented through the inhibition of the bacterial host (Torres-Barceló et al., 2014). In the case of lysogenic phages, it was also proposed that the antibiotics might induce the phage's lytic cycle, hence increasing its killing potential (Knezevic et al., 2013).

In addition to the enhancement of phages' killing effect, combining a small quantity of antibiotics with phages has the advantage of reducing the resistance apparition rate to both of the bactericidal agents compared to their individual application. This strategy exerts a heavy selective pressure on the targeted bacteria which induces a heavier fitness cost to them. This cost would drive resistant mutants eventually appearing towards being fitter to resist to the phage and antibiotic and less fit to reproduce and infect their hosts (Torres-Barceló et al., 2014; Jo et al., 2016).

The influence of the antibiotic resistance profile of a given bacteria on PAS is disputed, as examples showing the existence and absence of that influence can be found in the literature (Kamal and Dennis, 2015; Valério et al., 2017). Using sub MIC concentrations of antibiotic also allows to treat the phage-targeted bacteria, without the impact of a full antibiotic dose on the whole microbiota: this might be balanced by the eventually that the antibiotics used might also enhance naturally occurring phages' killing effect. This promising technique has given successful *in vitro* results with PP1131 phage cocktail, from the Phagoburn project (Oechslin et al., 2017). However, such use of antibiotics is not acceptable nowadays in terms of legislation for aquaculture which will delay the eventual broad release of commercial solutions.

4. Ecological consequences of massive phage use and food safety

4.1. Phage therapy and the environment

4.1.1. Ecological significance of marine bacteriophages

As the average concentration in surface coastal waters reaches 107 phage-like particles per mililitre, bacteriophages make the majority of virioplankton (Wommack and Colwell, 2000). They are responsible for the majority of procaryote mortality in the marine environment (Proctor and Fuhrman, 1990). They shape the bacterioplankton dynamics as they thrive on dominant bacterial populations and regulate them (kill the winner hypothesis) (Odelola and Koza, 1975; Hennes and Simon, 1995). The consequences of this active bacterial lysis propagate upwards in the trophic chain and alter carbon transfer from atmosphere to sediments and are hypothesized to eventually have effects on the climate as well (Fuhrman, 1992).

These results prove that phages' high numbers and key role in the marine trophic chain grant them a global impact. As the current understanding of microbial communities is limited, disrupting them with antimicrobials like phages could yield unforeseen global consequences. This should be kept in mind during the design of a product impacting these communities.

4.1.2. Environmental impact of phage therapy on fish farming areas

The three main known impacts phage therapy can have on the environment are genetic transfer through transduction, development of bacterial resistance and disruption of the microbiome. As mentioned earlier (in part 3) there are solutions to address the two first. Assessing the impact of phage therapy on the environment is especially important in our case since the aquatic environment acts as a vector for phages, allowing a quick dissemination.

Pereira et al. and Silva et al. studied the impact of single phage therapy on bacterial community structure. The phages described in their works targeted *Aeromonas salmonicida* and *Vibrio parahaemolyticus* and had a moderate impact on the overall bacterial community structure despite a broad host range which is an encouraging result (Pereira et al., 2011b; Silva et al., 2016). The time range of these two studies is however short and a repetition of these experiments at least over the course of a year should be conducted.

One may argue that the risk of disrupting environmental bacterial communities could be mitigated by using only the smallest necessary phages doses. However, as Meaden and Payne et al. discussed, phages pharmacokinetics are hardly predictable, especially compared to antibiotics. This is due to their ability to reproduce *in situ* and to the existence of an effectiveness threshold: if the phages are introduced in a small quantity their concentration might be below the critical point where they can effectively kill bacteria and the therapy will therefore fail. On the other hand, if they are able to reproduce freely, phages are likely to spread and persist in the environment (Meaden and Koskella, 2013; Payne and Jansen, 2003). This persistence is not ideal since it potentially provides the treatment with an impact outside of the aquaculture system.

Although we lack long term experience on environmental phage therapy, the majority of published work failed to highlight any major risk associated to phage-mediated microbial community disruption which is probably due to their host specificity. Despite their apparent harmlessness, it is important to test each and every phage's impact on the treated microbial community before using it at industrial scale.

4.2. How does phage-contaminated food impacts human health?

Bacteriophages are in general resistant to environmental stress and can persist all along the year in aquatic animals and can therefore be present in our meals (Comeau et al., 2005). Are they a threat to our health?

Phages are already very abundant in mammals' digestive track and thus are part of our normal microbiome, hence their presence is not abnormal in healthy individuals (Letarov et al., 2010). Most of the phages used in aquaculture phage therapy are furthermore isolated from the natural environment and are already ingested daily by people consuming seafood, without known detrimental effect. Also, phages used in an aquaculture setting will be aimed at specific pathogens which are not part of our normal microbiota and their ability to interact with it is therefore extremely weak, as is their ability to affect our health. Finally, phages are much more effectively translocated into the blood when administered intramuscularly than orally and are quickly eliminated from the bloodstream which lowers even more the risk of phage interaction with our body (Oliveira et al., 2009; Uchiyama et al., 2009). As a result, the impact of aquaculture phage use on human health seems very limited. Scientific literature is however lacking dedicated studies on the impact of phage-treated foods on our microbiota and general health, which would speed up the process of transferring phage therapy to the aquaculture industry.

5. Designing a phage-based product for aquaculture

5.1. Gathering the raw material: the bacteriophages

5.1.1. Where to search?

To seek a phage specific to a given bacteria, most authors recommend to search in the bacteria's ecosystem. Aquaculture farm water

Table 1
Examples of phage administration approaches considered for aquaculture.

Pathogen	Farmed species	MOI	Challenge	Phages used	Performance	Administration	Reference
Aeromonas salmonicida	*Solea senegalensis*	100	Immersion	AS-A	Mortality drop from 36–0%	Immersion	Silva et al. (2016)
Lactococcus garvieae	*Seriola quinqueradiata*	0,1	Injection	PlgY	Mortality drop from 90–45%	Injection	Nakai et al. (1999)
Lactococcus garvieae	*Seriola quinqueradiata*	0,1	Reverse gavage	PlgY	Mortality drop from 65–10%	Feed pellets	Nakai et al. (1999)
Pseudomonas plecoglossicida	*Plecoglossus altivelis*	1	Feed pellets	PPpW-3 PPpW-4	Mortality drop from 65–22%	Feed pellets	Park et al. (2000)
Pseudomonas plecoglossicida	*Plecoglossus altivelis*		Contaminated fish	PPpW-3 PPpW-4	Mortality drop from 90–26%	Feed pellets	Park and Nakai (2003)
Pseudomonas aeruginosa	*Clarias gariepinus*				Diameter of lesion from 15 mm to 5 mm	Swabbing	Khairnar et al. (2013)
Streptococcus iniae	*Paralichthys olivaceus*		Injection	PSiJ31 PSiJ32 PSiJ4 PSiJ42	Mortality drop from 80–0%	Injection	Matsuoka et al. (2007)
Vibrio anguillarum	*Salmo salar*	1	Immersion	CHOED	Mortality drop from 95–30%	Immersion	Higuera et al. (2013)
Vibrio anguillarum	*Salmo salar*	20	Immersion	CHOED	Mortality drop from 95–0%	Immersion	Higuera et al. (2013)
Vibrio harveyi	*Penaeus monodon*		Natural occurence	Viha10 Viha8	Mortality drop from 88–32% compared to antibiotic treatment	Immersion	Karunasagar et al. (2007)

and sediments, diseased animals or heavily contaminated ecosystems like sewage water are often used (Kim et al., 2012; Kim et al., 2010; Letchumanan et al., 2016; Kokkari et al., 2018; Surekhamol et al., 2014; Lal et al., 2017; Phumkhachorn et al., 2010). The rationale behind these strategies is that a phage is more likely to be present in its host ecosystem than anywhere else because of the specificity of phage-host interactions. Sewage water is a mixture of very rich and diverse ecosystems and is therefore an interesting place to search, even for aquaculture pathogens (Rong et al., 2014). This latter method is probably the fastest way to isolate a phage to control a new pathogen and has been evaluated for human phage therapy (Mattila et al., 2015).

In Gill and Hyman's review on the subject, they discussed that current methods used to search phages might favor the isolation of short-ranged phages (Gill and Hyman, 2010). Ecosystems displaying low bacterial concentrations could indeed select for wide host phages, while richer environments might stimulate the rise of highly specific phages. Although the information available on the subject is scarce, isolating phages from less dense bacterial ecosystems could help yielding broader-ranged phages.

As discussed in part 3.2.2, the search for new phages should be carried on regularly in order to compensate for the development of bacterial resistances. Some authors recommend to change phages every year for a given farm (Richards, 2014).

5.1.2. How to massively produce phages?

In a laboratory setting, phages are usually produced through a 4 step process:

- Production of a growing bacterial broth culture.
- Inoculation of phages and infection of the culture: phage replication step.
- The bacterial lysate is centrifuged to avoid clogging the filters used in the following step.
- Filtration of the supernatant: the filtrate is a cell-free lysate which contains the phages.

This traditional production process does however not comply with the safety and high quantity requirements of an industrial production: toxins (such as LPS) produced by the bacterial culture can persist in the end lysate and the centrifugation step is very costly for large volumes. Also, pathogenic propagation strains should be avoided to produce bacteriophages since their use in a factory would put workers at risk and induce some more safety costs. It is thus mandatory to use non-pathogenic surrogate hosts to produce phages aimed at pathogenic bacteria.

A few solutions (reviewed by Gill & Hyman) have been suggested to purify the phages from the bacterial culture and hence replace the centrifugation and filtration steps. For example, a precipitation, flocculation and low speed continuous flow centrifugation process can be used to treat several thousand litters of culture. Tangential flow filtration has also been used to purify large amounts of phages without any centrifugation step (Gill and Hyman, 2010).

Purification techniques such as chromatography are being developed to reduce endotoxin levels in the end phage suspension. Also, techniques already used to remove endotoxins from protein suspensions could be carried over to phage purification (Gill and Hyman, 2010; Smith and Gingrich, 2005; Boratyński et al., 2004; Smrekar et al., 2008). The minimum degree of purity of a given phage preparation depends on the intended use and cost and technical hurdles can be avoided if the purification steps are planned with the end use in mind: numerous fish species are for example resistant to endotoxins (Swain et al., 2008). As a result, it might not be necessary to completely remove endotoxins from phage preparation designed for these species. The required level of purity will also arguably be different between a product designed to be administrated via feed or via immersion.

5.2. Phage administration for industrial aquaculture

5.2.1. How to ideally use phages?

Different phage administrations approaches have been considered in the scientific literature for aquaculture: immersion, feed incorporation, injection and swabbing (Silva et al., 2016; Rong et al., 2014; Nakai et al., 1999; Christiansen et al., 2014). Table 1 gives a quick glance at a few studies displaying different modes of administrations for phages. For a better summary of published phage therapy trials, readers should consider reading the review of Gon Choudhury et al. (2017). Each approach has it pros and cons and it is difficult to point to an absolute best. To treat high volumes, immersion requires more phages which is costly but less time consuming compared to injection or intubation methods, which require fish by fish administration but allow a more parsimonious use of phages. Feed incorporation seems to be a fair compromise since it allows an easy treatment of all the individuals and provides a high concentration of phages for each of them without having to saturate the water with phages. This technique also allows a highly targeted application on digestive tract. The choice of the administration method also depends on the fish species, the nature of the infection and the characteristics of the phages. Parameters such as infected organs, ability of the phage to withstand gastric conditions and galenic processes or to cross the epithelial barrier dictate how they can be administrated (Richards, 2014; Prasad et al., 2011).

In most of published data, timing has proved to be a very important factor regarding phage therapy. Mortality typically increases heavily as treatment time reaches a few hours post infection (Jun et al., 2018; Lomelí-Ortega and Martínez-Díaz, 2014; Martínez-Díaz and Hipólito-Morales, 2013).The best choice seems to administer phages before the infection starts and continue thereafter, as a prophylactic and therapeutic treatment. This need for an early administration might be linked to the time necessary for the phages to reproduce and become sufficiently concentrated in order to make their host's population collapse before the bacterial infection reach an irreversible situation.

Although theoretical study tended to invalidate the following hypothesis, studying the influence of time and quantity of phages on the success of phage therapy could yield interesting results, as more concentrated administration might make up for a belated treatment (Payne and Jansen, 2003).

The success of phage therapy also relies on dosage. Although to the knowledge of the authors no aquaculture-focused data exists, it has been reported in aviculture that multiple successive phage administration can help increasing the success of phage therapy (Huff et al., 2003). We can here devise the dosages in three categories, ranked below in increasing concentration order:

- Too low to breach the efficacy threshold (Payne and Jansen, 2003).
- Active phage therapy.
- Passive phage therapy.

The efficacy threshold is the relative phage to bacteria concentration (Multiplicity Of Infection or MOI) below which phages cannot reproduce effectively thus being unable to impact their host's population. In active phage therapy, phages are concentrated enough to reduce the host's population through multiple reproduction cycles. Finally, passive phage therapy occurs when the quantity of administered phages is so high that the entire host population is lysed without needing any phage reproduction cycle. Passive phage therapy is obviously more costly, but has the advantage of allowing to circumvent all bacterial resistance mechanisms related to phage reproduction such as restriction modification and abortive infection, since such reproduction is not needed to provide a successful therapy (Cairns and Payne, 2008). The MOI required for a successful active phage therapy deeply vary among pathogen, fish species and phages. A MOI of 1 seems to be generally considered as an ideal value (Richards, 2014).

After deciding parameters such as time, quantity and administration path, a matching formulation is to be designed in order to effectively apply them.

5.2.2. Stabilizing and delivering phages thanks to galenics

Galenics and formulation directly impact how the phage-based products can be stored and their ability to reach their target once administered. In the peculiar case of surface infections like gill or skin lesions, targeting is effortless since these zones are directly accessible from the water. Phages also easily migrate from the digestive tract and water to the blood circulation system and all the organs (Christiansen et al., 2014).

As discussed in part 5.2.1, prophylactic use of phages followed by eventual therapeutic use seems to be the most efficient way to implement phage therapy. This requires frequent administration in order to keep the phage concentration close to therapeutically efficient levels. Galenics can bring a solution to this issue since phages can be encapsulated in order to be released over time thus reducing the number of administrations. In an aquaculture setting then, targeting the phages towards the right organs is not the main purpose of galenics. Instead the formulation is used to grant a longer shelf-life to the product and a longer persistence in the water thanks to a continuous release in the water (Malik et al., 2017).

Liquid and powder formulations seem to be the two most attractive formats for phages in aquaculture. Liquid formulations are especially easy to produce since they do not add any transformation step after the production of the phage lysate (Malik et al., 2017). However, phages are in general less stable in a liquid formulation and often require to be kept refrigerated. Only a small number of formulations for liquid phage product have been published, which is probably due to the stability issues of these methods (Ahiwale et al., 2013; Torres et al., 2014). Inspiration to design liquid formulations for phage might be found in liquid formulations for live virus vaccines for aquaculture, such as White's et al. contribution to the subject (White et al., 2016).

On the other hand, powders are more stable over time but require the application of energy consuming drying processes (Malik et al., 2017). They also have the advantage to be easily handled in numerous industrial processes. These formulations are produced using standard drying techniques, such as freeze drying, air drying, spray drying or spray freeze drying. The first constraint when choosing the treatment applied to phages is the ability of phage to survive its physicochemical conditions. Most of the phages are inactivated by temperatures higher than 75°C and are generally stable in at pH ranging from 5 to 8 (Pollard and Reaume, 1951; Krueger, 1932; Foster et al., 1949; Sharp et al., 1946; Putnam et al., 1949; Kerby et al., 1949). This temperature constraint necessitates adjustment of processes such as spray drying, which might then be performed at lower temperatures.

Encapsulation methods have been extensively reviewed by Malik et al. (2017). Briefly, methods such as emulsification followed by solvent removal or extrusion and gelation have been tested on fairly standard carrier polymers such as alginate, chitosan, polyvinylpyrrolidone and hydroxypropylmethylcellulose. Depending on the encapsulation method and polymer, different microparticles properties can be achieved such as release upon exposure to acids or degrading enzymes. Solutions involving liposomes and increasing the phage's ability to destroy unwanted biofilms have also been considered (Singla et al., 2016).

Just as phage antimicrobial effect varies from one phage to another with hardly predictable pattern, it is difficult to draw a general trend for phage formulation and processing. Some methods and excipient combination (such as freeze drying and disaccharides seem to yield good results in a lot of cases (Table 2). Each formulation remains to be tailored for each specific phage. This could turn as a serious issue when designing a formulation for a phage cocktail (Malik et al., 2017; Leung et al., 2017; Leung et al., 2018). Peculiarities of the farming environment should also be taken into account as formulation may also be used to mitigate the impact of variables such as radiation and salinity (Duarte et al., 2018; Silva et al., 2014b). Apart from the fact that larger viruses are more sensitive to recrystallization in powder formulation, no clear correlation has yet be found between phage phylogeny and recommended formulation (Vandenheuvel et al., 2014). It is important to note that this information applies to phages displaying an icosahedral capsid, which are the most studied. The best formulation published in present literature are those allowing for less than 1 log10 PFU (Plage

Table 2
Best performing formulations for phages according to Malik et al. (2017).

Method	Excipient	Conservation	Reference
Freeze drying	Peptides + Gelatine	25 °C	Engel et al. (1974)
Freeze drying	Peptides + Sucrose	25 °C	Engel et al. (1974)
Freeze drying	None	4 °C Or sub zero	Davies and Kelly (1969)
Spray drying	None	25 °C	Stanford et al. (2010)
Filter paper	Trehalose	4 °C	Colom et al. (2015)
Spray drying	Peptides + Trehalose	4 °C	Vandenheuvel et al. (2014)
Freeze drying	Peptides + Lactose	25 °C Or 4 °C	Golshahi et al. (2011)
Air drying	Peptides + Maltodextrin	25 °C Or 4 °C	Tang et al. (2013)
Liquid	Peptides + Maltodextrin	4 °C	Tang et al. (2013)

Forming Unit) titer loss during the process and less than 1 log10 PFU titer loss during the course of a year.

6. Conclusion

The present scientific, social and economic context is pushing bacteriophages back into the light. The interesting features of these viruses seem to grant them a place of choice in our future pool of antimicrobials. As public and private researchers are progressively removing the technical hurdles standing in front of global use of phage therapy, the major obstacles now lie in regulations. Phage libraries used for therapy and biocontrol should regularly be updated in order to match the evolving pathogens and thus do not fit in most countries' current regulatory framework where each phage would have to be registered and approved separately. This procedure currently makes the registration of a phage cocktail a feat and its periodical update a nightmare (Pelfrene et al., 2016; Verbeken et al., 2014; Verbeken et al., 2012). It should also be noted that more work is needed in order to rigorously prove the environmentally innocuousness and human health safety of phage treated foods, which are still holding authorities back from clearing phage therapy (ANSES, 2014).

The literature analyzed in this review enables us to draw a blueprint of the ideal phage in a concrete case, for example a phage-based solution to target vibriosis in shrimp ponds. This ideal phage would be:

- Able to kill all the pathogenic *Vibrio spp*...
- ... while being unable to kill beneficial *Vibrio spp* microbiota and other bacterial species.
- Exclusively lytic.
- Unable to perform transduction.
- Free of bacterial pathogenic and virulence genes.
- Displaying a slow resistance apparition rate.
- Easily produced and stored.

This ideal phage would be best used in closed environments with limited water exchanges such as Recirculating Aquaculture Systems (RAS) (Kalatzis et al., 2018).

Finally, the authors would like to underline the cautious approach that should be taken while introducing to the market or using a phage-based product namely regarding bacterial resistance and environment-related issues, in order to make the lessons of the antibiotic crisis flourish and avoid a phage crisis. Identification of the bacterial pathogens and adequate phage use should be preferred to blind administration of very wide host range cocktails. In order to ensure a sustainable use of phage therapy, protocols for efficacy and safety evaluation of the phages need to be legally adopted and made standard. Also, no therapeutic or prophylactic treatment should or could replace good hygiene practices, which are necessarily more economical and ecological: "better safe than sorry".

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Declaration of competing interest

The authors declare that they have no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2019.734423.

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