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Alternatives to antibiotics to control bacterial infections: luminescent vibriosis in aquaculture as an example

Tom Defoirdt[1,2], Nico Boon[1], Patrick Sorgeloos[2], Willy Verstraete[1] and Peter Bossier[2]

[1] Laboratory of Microbial Ecology and Technology (LabMET), Ghent University, Coupure Links 653, 9000 Ghent, Belgium
[2] Laboratory of Aquaculture and Artemia Reference Center, Ghent University, Rozier 44, 9000 Ghent, Belgium

The massive (mis)use of antibiotics to control infections in aquaculture has resulted in the development of resistant strains, which have rendered antibiotic treatments ineffective. Moreover, the horizontal transfer of resistance determinants to human pathogens and the presence of antibiotic residues in aquaculture products for human consumption constitute important threats to public health. Therefore, to make the aquaculture industry more sustainable, new strategies to control infections are urgently needed.

Introduction

Aquaculture comprises all forms of culture of aquatic animals and plants in fresh, brackish and marine environments [1]. Reports by the United Nations' Food and Agriculture Organization (FAO) consider disease outbreaks as a significant constraint to the development of the aquaculture sector, with a global estimate (made by the World Bank in 1997) of disease losses in the range of US$3 billion per year [2]. Here, we focus on disease caused by luminescent vibrios (i.e. *Vibrio harveyi* and closely related bacteria such as *Vibrio campbellii* and *Vibrio parahaemolyticus*). These bacteria belong to the *Gamma-proteobacteria* and are Gram negative, usually motile rods [3]. Although these bacteria are commonly denoted as luminescent vibrios, not all strains are luminescent [4,5]. Vibrio disease is described as vibriosis or bacterial disease, penaeid bacterial septicemia, penaeid vibriosis, luminescent vibriosis or red-leg disease [6]. Signs of disease include lethargy, tissue and appendage necrosis, slow growth, slow metamorphosis, body malformation, bolitas negricans, bioluminescence, muscle opacity and melanization [6]. In many cases, vibrios are opportunists, only causing disease when the host organism is immune suppressed or otherwise physiologically stressed, with the frequency of infection often being attributable to intensive culture and adverse environmental conditions [7].

Almost all types of cultured animals can be affected by these bacteria (Table 1). However, the most serious problems have been reported in penaeid shrimp culturing, and luminescent vibriosis has become a major constraint on shrimp production in South America and Asia [8]. The losses due to luminescent vibriosis in Indonesian hatcheries in 1991, for instance, have been reported to be as high as US$100 million [9]. In addition to affecting cultured animals, luminescent vibrios can also cause human infections, and related bacteria, such as *Vibrio cholerae* and *Vibrio vulnificus*, are known to be serious human pathogens [3].

Antibiotics: the ambivalent solution

Traditionally, antibiotics have been used in attempts to control bacterial disease in aquaculture. For example, Holmström *et al.* [10] reported that, of the 76 shrimp farmers they interviewed in Thailand, 56 used antibiotics. Most of those farmers used the antibiotics prophylactically, some on a daily basis. More than ten different antibiotics were used, including chloramphenicol, gentamycin, trimethoprim, tiamulin, tetracyclines, quinolones and sulfonamides. Moriarty [11] estimated the use of antibiotics in shrimp farm production in Thailand to be as high as 500–600 tonnes in 1994. In the Philippines, oxytetracycline, oxolinic acid, chloramphenicol, furazolidine, nitrofurans, erythromycin and sulfa drugs are commonly used to treat bacterial diseases [12]. Chemotherapy is also widely practiced in South America. In Mexico, for instance, the most commonly used antibiotics in shrimp farms are oxytetracycline, florfenicol, trimethoprim-sulfamethoxazole and, more recently, sarafloxacin and enrofloxacin [13].

In view of the massive (mis)use of antibiotics in aquaculture, it is not surprising that many reports have mentioned (multiple) resistance of luminescent vibrios to several antibiotics (Table 2). Consequently, currently, antibiotics are no longer effective in treating luminescent vibriosis. Karunasagar *et al.* [14], for instance, reported mass mortality in black tiger shrimp (*Penaeus monodon*) larvae caused by *V. harveyi* strains with multiple resistance to cotrimoxazole, chloramphenicol, erythromycin and streptomycin. Of these antibiotics, the first two had been regularly used as prophylactics.

Apart from rendering treatments ineffective, the excessive (mis)use of antibiotics in aquaculture also constitutes a direct threat to human health and to the environment [7,15]. The antibiotic resistance determinants that have emerged and/or evolved in the aquaculture environments have been shown to be transmitted by horizontal gene transfer to bacteria of the terrestrial environment, including animal and human pathogens. For example, the

Corresponding author: Defoirdt, T. (tom.defoirdt@ugent.be).
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Table 1. Diseases of cultured animals caused by *V. harveyi* (including its junior synonym *V. carchariae*)

Host organism	Disease	Refs
Crustaceans		
Brine shrimp (*Artemia franciscana*)	Between 45% and 80% mortality	[75]
Kuruma prawn (*Penaeus japonicus*)	High mortality without overt gross signs	[76]
Ridgeback prawn (*Sicyonia ingentis*)	Detachment of the midgut epithelium, resulting in up to 55% mortality	[77]
Rock lobster (*Jasus verreauxi*)	Luminescent vibriosis with up to 75% mortality in phyllosoma larvae	[78]
Tiger prawn (*P. monodon*)	Luminescent vibriosis resulting in mass mortality	[14,79]
White shrimp (*Litopenaeus vannamei*)	Up to 85% mortality in nauplii	[80]
Fish		
Cobia fish (*Rachycentron canadum*)	Gastroenteritis followed by mass mortality	[81]
Grouper (*Epinephelus coioides*)	Gastroenteritis followed by mass mortality	[82]
Red drum (*Scaienops ocellatus*)	Gastroenteritis followed by mass mortality	[83]
Salmonids	Up to 100% mortality	[84]
Seahorse (*Hippocampus* sp.)	Hemorrhages resulting in more than 90% mortality	[85]
Summer flounder (*Paralichthys dentatus*)	Necrotizing enteritis	[86]
Molluscs		
Abalone (*Haliotis tuberculata*)	Between 60 and 80% mortality	[87]
Japanese abalone (*Sulculus diversicolor*)	Mass mortality	[88]
Pearl oyster (*Pinctada maxima*)	Mass mortality	[89]

V. cholerae that caused the 1992 Latin-American epidemic of cholera seemed to have acquired antibiotic resistance as a result of coming into contact with antibiotic-resistant bacteria selected through the heavy use of antibiotics in the Ecuadorian shrimp industry [16]. The presence of residual antibiotics in commercialized aquaculture products constitutes another problem with respect to human health because this can lead to an alteration of the normal human gut microflora and can generate problems of allergy and toxicity [15]. Given the world-wide trade in aquaculture products, health problems related to antibiotic use in aquaculture are not limited to producing countries, but are also relevant to importing countries.

It might be clear from the above that global efforts are needed to promote more judicious use of antibiotics in aquaculture and that new strategies to control pathogenic bacteria are needed to make the industry more sustainable. Currently, measures to protect aquaculture animals from luminescent vibriosis without using antibiotics are being developed and tested. A holistic approach, which includes environment, host and pathogen, will probably be most sustainable (Figure 1). In this new view on biocontrol, measures that prevent disease are the most important health management option [2]. However, it is not always economically feasible to culture the animals in the most optimal conditions, so there will always be a risk to infection and a need for effective biocontrol techniques. Alternative biocontrol measures directed towards luminescent vibrios that have recently been developed will be discussed throughout the following paragraphs; measures that aim at disease prevention and disease control at the level of the host are briefly discussed in Boxes 1 and 2.

Killing the vibrios: phage therapy

In the early 1920s, bacteriophages were discovered as viral infections of bacteria, and their value for antibacterial therapy and prophylaxis was almost immediately recognized. Surprisingly, phages were only relatively recently proposed as candidate therapeutics for aquaculture [17]. Several reports described the isolation of phages of luminescent vibrios, including lysogenic ones [18] and more recently also lytic phages [19–21]. We refer to previously published reviews for detailed information about phage therapy in general [17,22,23]. A major advantage of phage therapy is that non-target microbiota are not affected because the phages usually have a narrow host range [17]. However, many phages are strain specific rather than species specific [17], so phages for use as biocontrol agents to treat luminescent vibriosis should be selected on their capability to infect a wide range of luminescent vibrios (Box 3).

Attempts to use phages to control luminescent vibriosis have only recently been reported. Shivu *et al.* [21] isolated seven phages from hatchery and creek water and tested their lytic spectrum against 183 *V. harveyi* strains originating from different geographical regions. They found that the phages lysed between 15% and 69% of the strains. None of the phages was able to infect other *Vibrio* species. The authors concluded that shrimp hatcheries would be a good source for the isolation of phages to be

Table 2. Examples[a] of multiple antibiotic resistance in *V. harveyi* and closely related bacteria isolated from aquaculture facilities in Asia and South America

Location	Antibiotics	Refs
India	Cotrimoxazole, chloramphenicol, erythromycin and streptomycin	[14]
Java	Tetracyclin, ampicillin and other β-lactams	[90,91]
Mexico	Ampicillin, amikacin, carbenicillin, cephalotin and oxytetracycline	[92]
Philippines	Oxytetracycline, furazolidone, oxolinic acid and chloramphenicol	[12]
Philippines	Kanamycin, gentamycin, carbenicillin and ampicillin	[93]
Taiwan	Nitrofurantoin, novobiocin and sulfonamide	[94]
Thailand	Kanamycin and carbenicillin	[93]

[a]This is an overview of resistance data published in the literature. Data in the literature regarding other countries (e.g. China and Ecuador) were not available.

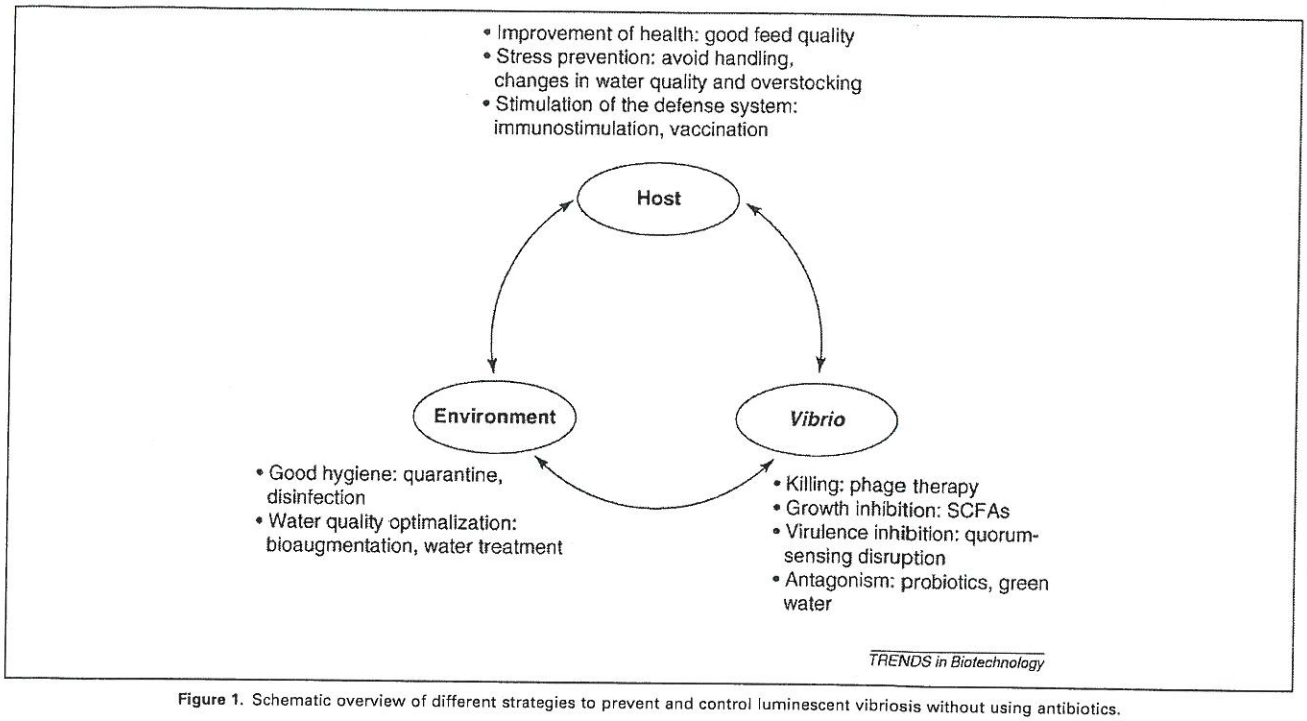

Figure 1. Schematic overview of different strategies to prevent and control luminescent vibriosis without using antibiotics.

used as therapeutics. Vinod *et al.* [20] isolated a phage from shrimp farm water with lytic activity against all 50 *V. harveyi* isolates tested. The phage was tested both in a laboratory system and in a hatchery for its potential to protect *P. monodon* larvae. In the laboratory system, addition of the phage increased the survival of shrimp larvae infected with pathogenic *V. harveyi* by 45–55% and decreased luminescent bacterial counts by 2–3 log units after two days. In the hatchery trial, addition of the phage increased shrimp survival from 17% to 86% after 17 days. Interestingly, the phage treatment performed much better than daily addition of antibiotics (5 mg/l oxytetracyclin and 10 mg/l kanamycin), in which the shrimp survival was only 40%.

Box 1. Disease prevention

According to the FAO, disease prevention is the preferred health management option with respect to aquaculture diseases because preventive measures are most cost-effective [2]. Disease prevention can be achieved by improved management, including the prevention of the transmission of pathogens between farms (e.g. by quarantine [2]), and good hygiene (e.g. the disinfection of culture tanks, water and eggs [60]). In many cases, aquaculture pathogens are opportunists, only causing disease when the host organism is immune suppressed or otherwise physiologically stressed [7], so eliminating stress is also an important factor that can reduce infections. This could be achieved by improving the water quality by water treatment [61] and bioaugmentation [62] and avoiding important stress factors such as high stocking densities, handling, temperature and salinity changes [60]. Furthermore, it seems that the feed quality also has an important impact on the susceptibility of cultured animals to luminescent vibriosis infections, so that animals that are in better physical health are more resistant to infection [63]. However, it is not always economically feasible to culture the animals in the most optimal conditions and give them the optimal feed, so there will always be a risk of infection and a need for effective biocontrol techniques.

A constraint to the use of phages as therapeutics is that phages can transfer virulence factors [18,19]. Hence, before using bacteriophages for therapy, it will be important to test whether they carry any virulence genes and whether they would be safe to use. A second important problem is the rapid development of resistance to phage attachment, which renders bacteria resistant to phage attack [23]. This problem could be overcome by applying cocktails of phages or by using phage components instead of intact phage.

Growth inhibition: short-chain fatty acids

Alternative strategies to control luminescent vibriosis could aim at inhibiting growth of the pathogens rather than killing them. Short-chain fatty acids (SCFAs) are known to inhibit the growth of pathogenic bacteria. Coated and uncoated SCFAs are currently used in commercial animal diets to control pathogens such as *Salmonella* [24]. Based on this, Defoirdt *et al.* [25] investigated whether these compounds could also be used to control luminescent vibriosis. *In vitro* tests showed that SCFA inhibited the growth of pathogenic luminescent vibrios in liquid medium. The effective growth-inhibitory SCFA concentrations were in the millimolar range and were dependent on the pH of the medium, with approximately ten times more SCFA needed per unit pH increase. Furthermore, *in vivo* challenge tests with gnotobiotic brine shrimp (*Artemia franciscana*) showed that 20 mM (≈2 g/l) of formic, acetic, propionic, butyric or valeric acid increased the survival of nauplii infected with a virulent *V. campbellii* strain from 20% to ~45%, with no difference between the different SCFAs. In addition to this, Vázquez *et al.* [26] recently reported that SCFAs (and not bacteriocins) are responsible for the inhibitory effect of lactic acid bacteria towards pathogenic vibrios.

Box 2. Vaccination and immunostimulation

Stimulation of host defenses can increase resistance to infectious disease by triggering specific immune responses (i.e. by vaccination) in addition to enhancing nonspecific defense systems (i.e. by immunostimulation) [64]. Immunostimulants receiving most attention comprise live bacteria, killed bacteria, glucans, peptidoglycans and lipopolysaccharide [65]. Vaccination refers to the administration of weakened or dead pathogenic bacteria or parts of them, with the aim of conferring long-lasting protection through immunological memory [66]. Vaccination requires primary challenge with antigen and relies on specific defense mechanisms which are traditionally believed only to exist in vertebrates; consequently, with respect to aquaculture, vaccination would only be possible in fish and not in crustaceans [65]. However, there has been some evidence that invertebrates also possess adhesion immunoglobulin superfamily molecules, which in mammals are known to be involved in adaptive immune responses [67]. We refer the reader to previously published reviews for more general information on immunostimulation [65,68] and vaccination [66] in aquaculture.

The effects of vaccination against luminescent vibriosis in several fish species have been studied by different research groups, with promising results. Elevated antibody and *Vibrio* inhibitory activities, induction of immune memory and significantly increased survival of experimentally infected fish have been reported [69–71]. Unfortunately, vaccination is not possible in the case of fish larvae (which generally are most susceptible to disease) because it is practically infeasible to handle these small animals and, more importantly, because it is believed that fish larvae do not have the ability to develop specific immunity [68].

Several reports have mentioned the use of immunostimulants to control luminescent vibriosis in shrimp, resulting in increased prophenoloxidase and phenoloxidase activities and hemocyte counts, and significantly increased survival after experimental infection with luminescent vibrios [72–74]. Although immunostimulation shows promise, there are some limitations that should be considered. First of all, immunostimulation might be too intense and can harm or even kill the host [65,68]. Second, because there is no memory component involved, the response is likely to be short in duration, and hence immunostimulants will have to be administered repeatedly [64]. However, long-term administration of such agents seems to decrease the immunostimulant effect and does not always promote disease resistance [64,65].

Box 3. Summary of alternative biocontrol measures directed towards luminescent vibrios

Phage therapy

Positive aspects:
- Specific killing of pathogens
- No effect on harmless and beneficial microbiota

Negative aspects:
- Resistance development by alteration of phage attachment sites
- Too narrow host range
- Possible transfer of virulence factors

Possible solutions:
- Careful screening
- Use of phage cocktails or phage components instead of intact phage

SCFAs and PHB

Positive aspects:
- Effective in *Artemia* model system
- Use of PHB is economically attractive

Negative aspect:
- Effectiveness needs to be confirmed in other host systems

Quorum-sensing disruption

Positive aspect:
- No selective pressure, so there is a low risk of resistance development

Negative aspect:
- Technology for practical applications is not yet available

Probiotics

Positive aspect:
- Takes advantage of natural antagonism: different modes of action possible

Negative aspect:
- High doses needed, in many cases not able to maintain themselves

Possible solutions:
- Selection procedure: different modes of action, isolation from system in which they will be applied

Green water

Positive aspect:
- Control measure is part of the system (integrated aquaculture)

Negative aspect:
- Effectiveness in controlling disease still has to be proven

Apart from inhibiting the growth of unwanted bacteria, SCFAs are also known to be a preferred source of energy for the colonic mucosa in mammals and to increase the health of the gastrointestinal epithelium [27]. It is not yet clear whether this is also true for aquatic animals, although Weltzien *et al.* [28] reported that brine shrimp can use the SCFA β-hydroxybutyrate as an energy source. This would mean that SCFAs not only function at the level of the pathogen, but also affect the host beneficially, and this might result in higher resistance to disease.

The fact that effective SCFA concentrations needed to protect brine shrimp from luminescent vibriosis were relatively high was attributed to the fact that brine shrimp are particle filter-feeders and cannot accumulate dissolved compounds [25]. Consequently, it was reasoned that the efficiency of these compounds might be increased by dosing them in particle form to the culture water. Based on literature reports mentioning that poly-β-hydroxyalkanoate polymers can be degraded into β-hydroxy SCFAs, and on the observation that β-hydroxybutyrate has the same positive effect towards infected brine shrimp as do other SCFAs, Defoirdt *et al.* [29] started to investigate whether poly-β-hydroxybutyrate (PHB) could be used as an elegant method to deliver SCFAs to the brine shrimp gut. The addition of 1000 mg/l commercial PHB particles (average diameter 30 μm) to the culture water offered a complete protection (no significant mortality when compared with uninfected nauplii) from the pathogenic *V campbellii* [29]. In a second study, it was shown that the addition of 10^7 cells/ml of PHB-containing *Brachymonas* bacteria (corresponding to ~10 mg/l PHB) also completely protected the shrimp from the vibrios [30]. Although the exact mode of action is still unclear, it was hypothesized that the PHB polymer is (at least partially) degraded to β-hydroxybutyrate in the *Artemia* gut and that the release of this SCFA protects the shrimp from the pathogen.

Although these experiments should be repeated in other host systems, they suggest that PHB addition to the culture water or feed could be an interesting biocontrol measure. Indeed, PHB can be produced relatively easily [31]. Moreover, PHB production on waste streams (such as

Finally, several human pathogens, including *V. cholerae* and *V. vulnificus*, have been found to contain a quorum-sensing system that is similar to the *V. harveyi* quorum-sensing system [46]; consequently, quorum-sensing-disrupting techniques developed to control luminescent vibriosis might also be useful to treat infections caused by these human pathogens.

Antagonism: probiotics

Interest in the application of probiotics in aquaculture is fairly recent. Possible modes of action that have been mentioned in the literature for probiotics include (i) production of inhibitory compounds, (ii) competition for nutrients, (iii) competition for adhesion sites in the gastrointestinal tract, (iv) enhancement of the immune response and (v) production of essential nutrients such as vitamins and fatty acids, and enzymatic contribution to digestion [47,48]. In addition to this, several authors also considered bacteria that improve the water quality by removing toxic inorganic nitrogen or by mineralizing organic matter as probiotics [47]. Based on the observations that bacterial cell-to-cell communication regulates the virulence of luminescent vibrios (see earlier), another mode of action of probiotics could be specific inhibition of virulence gene expression, for instance by disrupting cell-to-cell communication.

Most investigations on probiotics as biocontrol agents to treat luminescent vibriosis have been performed using *Bacillus* strains. In one of the earliest reports, Moriarty [49] found that the addition of a mixture of *Bacillus* strains that had been selected for the production of antibiotics against luminescent vibrios resulted in healthier prawns and lower numbers of luminescent vibrios in the pond water. Rengpipat *et al.* [50] found that the addition of *Bacillus* strain S11 to *P. monodon* infected with a pathogenic *V. harveyi* strain increased survival of the shrimp from 26% to 100% after ten days in laboratory-scale experiments. By contrast, later experiments showed only marginal increases in the survival of challenged shrimp in farm trials following addition of the *Bacillus* strain to the feed [51]. Vaseeharan and Ramasamy [52] showed that *Bacillus subtilis* strain BT23, isolated from shrimp culture ponds, produced a substance which inhibited the growth of *V. harveyi*. The addition of the *Bacillus* strain to the culture water of black tiger shrimp larvae resulted in a 90% decrease in accumulated mortality after 15 days.

Recently, Decamp *et al.* [53] reported some field data of the use of a commercial mixture of *Bacillus* strains, selected on their ability to inhibit pathogenic vibrios, to grow under hatchery conditions and to degrade waste products, on the performance of shrimp. In a Thai hatchery, the addition of the mixture to the culture water significantly improved the survival of *P. monodon* larvae and performed equally well as antibiotics. Similar results were obtained in a Brazilian hatchery with *Litopenaeus vannamei* larvae. Unfortunately, statistical analyses were not possible in the latter case owing to a lack of replicates.

The most important limitation to the use of probiotics is that in many cases they are not able to maintain themselves, and so need to be added regularly and at high concentrations [48], which makes this technique less cost-effective. Moreover, probiotics that were selected *in vitro* based on the production of inhibitory compounds might fail to produce these compounds *in vivo* [47,48]. Finally, the vibrios might develop resistance if the production of growth-inhibitory compounds is the only mode of action, as has occurred for numerous antibiotics. From the above, it seems to be clear that selection of probiotics needs to be performed carefully and that it might be advantageous to isolate candidate probiotics from the culture system(s) in which they will be applied because, in this case, the chance that they will be able to establish themselves is expected to be higher [47]. Moreover, it might be beneficial to select for probiotics with more than one antagonistic characteristic or to apply a mixture of probiotics with different modes of action, to maximize the chance of success. Alternatively, from a commercial point of view, it might be more advantageous to investigate whether probiotics that are already licensed for use in human or animal nutrition could be used instead of isolating new probiotics because licensing new probiotics for use in animal products is relatively expensive [54] and this might limit the commercial development of new probiotic products.

Antagonism: green water

In the so-called green water technique, shrimp are cultured in water from tilapia ponds, in which microalgae (such as *Chlorella*) grow abundantly. Tendencia and dela Peña [55] reported that *V. harveyi* disappeared from seawater containing *Chlorella* after two days of incubation. More recently, Lio-Po *et al.* [56] identified eight bacterial and 12 fungal isolates that were associated with green water and that had a promising growth-inhibitory effect towards luminescent *V. harveyi*. Interestingly, the majority of the luminescent *Vibrio*-inhibiting bacteria were isolated from tilapia skin mucus and gut. *V. harveyi* was also found to be inhibited by several microalgae associated with the green water. A 3-log decrease in luminous *Vibrio* counts was observed in coculture with the microalga *Leptolyngbya* sp. after one day of incubation. For the algae *Chaetoceros calcitrans* and *Nitzchia* sp., an even higher growth-inhibitory effect was noticed because luminous vibrios completely disappeared from the (co-)cultures after one and two days, respectively. Unfortunately, as far as we know, to date, no experimental data have been reported that demonstrate that green water or algae can indeed protect cultured animals against luminescent vibriosis disease. Moreover, more research will be needed to elucidate further the mechanism by which green water decreases luminescent *Vibrio* levels.

Conclusions and further perspectives

This review aimed at providing a critical evaluation of alternative measures that have recently been developed to control disease caused by *V. harveyi* and closely related bacteria. Techniques discussed include phage therapy, the use of SCFAs and polyhydroxyalkanoates, quorum-sensing disruption, probiotics and green water. Some of the techniques have only been studied recently and have only been tested in the laboratory (e.g. disruption of cell-to-cell communication), whereas others have a longer history, including farm trials (e.g. the application of probiotics).

Each of the techniques has its advantages but also its limitations. In fact, none of them will probably be successful in all cases. Therefore, it is of importance to develop further all of these alternatives to construct a toolbox containing different sustainable biocontrol measures. A good biocontrol management strategy might then use different techniques in rotation to prevent resistance development. Alternatively, it might be valuable to determine which techniques are, and which are not, compatible with each other, to apply them together to maximize the chance of protecting the animals successfully. Finally, some of the alternative techniques that are being developed to control luminescent vibriosis in aquaculture might also be useful in treating human infections caused by related bacteria such as *V. cholerae* and *V. vulnificus*.

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