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Provisional chapter

The Use and Benefits of *Bacillus* Based Biological Agents in Aquaculture

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Additional information is available at the end of the chapter

1. Introduction

Global shortages in seafood resources have driven the growth of aquaculture as an economic activity, predominantly in developing countries [1-2]. As a consequence of space and resource constraints, traditional aquaculture has been intensified into reticulated systems with high stocking densities of the cultured species [3-4]. This results in an artificial environment that has a propensity for supporting the growth of pathogenic bacteria and the accumulation of waste metabolites in aquaculture systems [5]. The indiscriminate release of spent aquaculture wastes into surrounding environments is also problematic [6-7].

The outbreak of disease in aquaculture systems, caused by bacterial pathogens, is a complex phenomenon associated with stressful environmental conditions such as poor water quality and can ultimately result in mass mortality and significant loss to the industry [8-9]. The main cause of poor water quality is waste accumulation through hyper-nutrification resulting from excessive feeding rates and high nutrient dietary composition, both of which are common phenomena in intensive aquaculture systems [13-15]. High levels of nitrogenous and phosphorous waste accumulation predispose fish to infestation by parasites and pathogens and also pose a threat to the environment [13,16-17]. Selection for certain characteristics by breeders has also in some cases reduced the vigour in breeding lines, making fish less hardy and more susceptible to disease [10]. Of particular importance is the prevalence of bacterial disease, which results in damage and often leads to death of fish [11]. Gram-negative bacteria such as *Aeromonas hydrophila* are amongst the main pathogenic micro-organisms responsible for bacterial disease [8,12]. Conventional methods of dealing with disease include the use of chemicals and antibiotics, which alter natural microbial populations, damage the environment and increase resistance and virulence of pathogenic micro-organisms [5,17-21].

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Useful micro-organisms play a number of roles in pond culture, particularly with respect to productivity, nutrient cycling, nutrition of the cultured animals, water quality, disease control and environmental impact of effluents [22-24]. Bacterial additives demonstrate the potential to improve water quality and reduce pathogen load and mortality, and have thus emerged in modern day aquaculture as alternatives to chemicals and antibiotics [17,24]. Many bacterial strains have also demonstrated a significant algaecidal effect, which is advantageous in aquaculture systems through reduction of algal growth and hence algal blooms which can destabilise these systems [25-26]. Biological agents such as Gram-positive *Bacillus spp.* offer an attractive solution to the challenges facing modern aquaculture. Advantages of this genus include the ability to grow rapidly, tolerate a wide range of physiological conditions and the ability to sporulate. The robust spores of *Bacillus spp.* are also amenable to simple and cost effective production processes and the end products are stable for long periods [24, 27].

2. Aquaculture as an economic activity

The Food and Agriculture Organization of the United Nations [28] reported that capture fisheries and aquaculture supplied the world with about 154 million tonnes of fish in 2011, of which 131 million tonnes were used for human consumption [28]. Aquaculture contributed 79 million tonnes to the global fisheries market in 2010 at a value of $125 billion. Aquaculture farming used for food consumption comprised 60 million tonnes ($119 billion), 15 million tonnes was used for fish meal and fish oil production, while the remainder was used for ornamental fish production. With sustained growth in fish production and improved distribution channels, world supply of fish for human consumption has grown dramatically in the last five decades. An average growth rate of 3.2% per year in the period 1961–2009, has outpaced the increase of 1.7% per year in the world's population. The global aquaculture market comprises both marine and inland (freshwater) farming. The majority (90%) of fresh water ornamental fish are captive bred, compared to only 25 of the 8000 species of marine fish. In 2010, 75% of the quantity of fish and fishery products produced consisted of products destined for human consumption, with ornamental aquaculture contributing a smaller volume.

Aquaculture production is dominated by developing countries, and predominates in Asian countries. The methods of practice of aquaculture have evolved into intensive reticulated systems, in contrast to traditional extensive systems, due to restrictions in availability of land and as a consequence of increased environmental awareness. Aquaculture is probably the fastest growing food-producing sector globally, and the most recent estimates for worldwide aquaculture show that it contributes just over 50% of total fish production. This has been an astonishingly fast growth rate from only 16% of total consumption 15 years ago. The key impetus for growth of the market is global food security and a resistance towards resource exploitation through over-harvesting of natural waters [29]. The consumer drives the aquaculture practice, product quality and branding. End products must thus address consumer food concerns and must at least be as desirable as naturally harvested products.

3. Current challenges of the aquaculture industry

Key challenges to the development and growth of aquaculture as an economic activity are limited water resources, energy requirements and the environmental impact of aqua-farming methods. To address these challenges water is re-cycled and farming activities are intensified, resulting in an increase in stocking density, deterioration in water quality, increased incidence of disease, poor feed to body mass conversion efficiencies and higher mortality rates. The net result is reduced yield. Annual losses to the market due to disease, water quality and nutrition are estimated at 40% [30].

3.1. Disease in aquaculture

Definitions of disease include an unhealthy condition or infection with a pathogen. Disease is a complex phenomenon, leading to some form of measurable damage to the host [12]. Outbreaks of disease either begin suddenly and progress rapidly, often with high mortalities, and disappear with equal rapidity (acute disease) or develop more slowly with less severity, but persist for greater periods (chronic disease). Fish disease is the outcome of aberrations to the delicate interaction between the hosts, the disease-causing agent, and external conditions such as unsuitable changes in the environment, poor hygiene and overcrowding. Disease outbreak is generally associated with a primary invasion by parasites or mechanical injury, coupled to stressful environmental conditions such as changing temperature and poor water quality [8]. The prevalence of infectious agents can result in mass mortality causing significant losses to aquaculture operations [9]. Fish diseases such as rotting fins and ulceration of the skin are more prevalent when fluctuation in temperature causes immuno-modulation, resulting in inferior disease resistance and increased mortality [31-32]. An array of stress factors such as poor water quality, parasite load or a natural physiological state (e.g. during the reproductive phase) in the life cycle of the fish are also often associated with outbreaks of disease [12]. Strict selection for desirable characteristics by breeders has also reduced the vigour in breeding lines, making fish less hardy and more susceptible to disease [35]. Disease is not necessarily caused by the action of a single bacterial taxon, as representatives of many bacterial taxa have at one time or another been associated with disease outbreaks. *Aer. hydrophila* and *Pseudomonas spp.* are among the predominant species responsible for causing fish diseases [33]. Many bacterial pathogens are members of the normal microflora of water and/or fish. However not all of these bacteria are primary pathogens as many can be categorized as opportunistic pathogens, which colonize and cause disease in already damaged hosts.

Environmental factors play a key role in the onset of disease which is reported as being a consequence of the interaction between the host, environmental stress and prevalence of disease causing agents [8,12,34]. Some diseases are prevalent in spring and associated with environmental change to warmer temperatures, a period which is also characterised by an increase in the activity of pathogenic bacteria and parasites. Temperature fluctuation causes transient immuno-modulation of fish, which can result in reduced disease resistance [31-32]. Haemorrhagic septicaemia is an example of this phenomenon, with the disease resulting from infection by a wide range of pathogens that cause open ulcerated lesions and haemorrhages

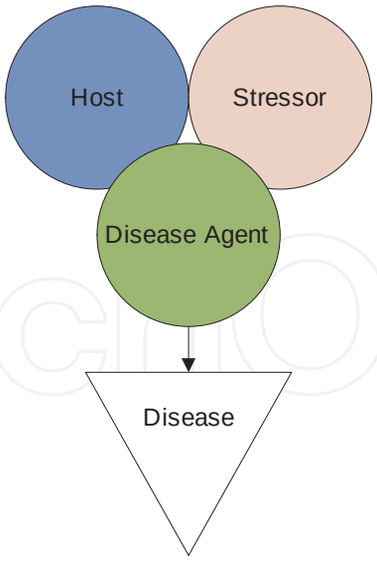


Figure 1. Interactive factors influencing disease in aquaculture (modified from [12]).

on the infected fish [12,36-37]. Additional clinical symptoms can include fin and tail rot, the loss of scales, localized haemorrhages, particularly in the gills and vent, exophthalmia and abdominal distension [12]. The acute form of this disease is of sudden onset, and the fish usually die within 2-3 days [38-40]. The main pathogenic micro-organisms involved in septicaemia are *Aer. hydrophila, Aer. salmonicida*, and to a lesser extent *Pseudomonas flourescens* [8,12]. *Aer. hydrophila* is known to produce haemolysin, cytotoxins and enterotoxins which cause tissue necrosis resulting in ulcers, dropsy and abdominal oedema associated with haemorrhagic septicaemia [8]. *Aer. salmonicida* has been specifically associated with ulcerative erythrodermatitis and furunculosis [8,12,41]. *P. flourescens*, which is ubiquitous in fresh water and is generally regarded as a secondary invader of damaged tissue, has also been associated with outbreaks of septicaemia [42-45]. There is therefore merit in reducing the prevalence of these bacteria in aquaculture systems.

Pathogen	Disease
Aeromonas hydrophila	Haemorrhagic septicaemia, motile *Aeromonas* septicaemia, redsore disease, fin rot
Aeromonas salmonicida	Furunculosis, carp erythrodermatitis, ulcer disease.
Pseudomonas fluorescens	Generalized septicaemia
Pseudomonas pseudoalcaligenes	Skin ulceration

Table 1. Predominant bacterial pathogens causing disease of *Cyprinus carpio* (modified from [12]).

3.2. Water quality

Use of reticulated systems for intensive culture results in substantial amounts of particulate organic and soluble inorganic excretory waste, due mainly to increased stocking density [17]. The main source of this waste is hyper-nutrification, resulting from excessive feeding rates and

high nutrient dietary composition, which has a significant influence on the survival, growth and reproduction of fish [13-15,17,46]. Nitrogen and phosphorous waste accumulation pose a threat to the environment and can predispose fish to infestation by parasites and pathogens due to a reduction in immunity [13,17].

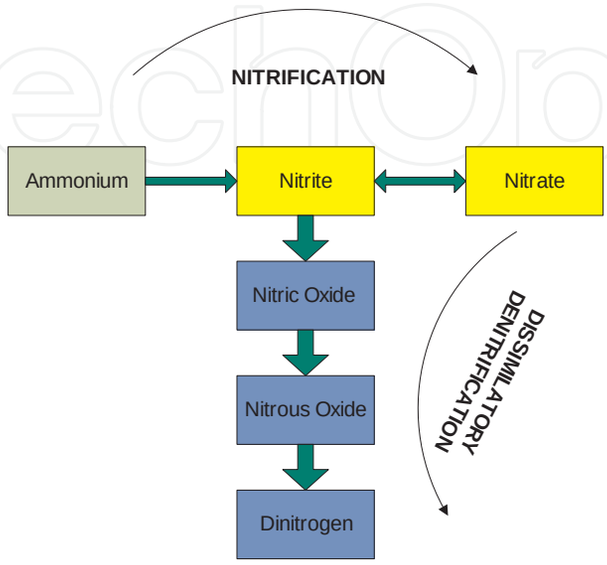


Figure 2. Nitrification and denitrification cycle [30].

Ammonia is a primary metabolic waste of fish and is excreted through the gills by bronchial diffusion. It is also produced by bacterial ammoniafication of uneaten food and faeces and is released from the mineralization of sediment [47-50]. Ammonia is oxidised to nitrite and finally to nitrate through the process of nitrification, with ammonia and nitrite being the most toxic of these metabolites to fish. Nitrite can also be produced through the process of denitrification [48]. Ammonia concentrations above 0.3 mg/l have been reported as toxic to fish, with hyperplasia of gill tissue, gill necrosis, pathological evidence of kidney and liver damage and reduction in growth rate occurring at this and higher concentrations [51-53]. Exposure to high ammonia concentration also causes epithelial lifting on gill filaments resulting in respiratory impairment and mortality [54]. Nitrite is usually present at low concentrations in natural systems, except when there is an imbalance, because it is a common intermediate in nitrification and denitrification, catabolic ammoniafication and nitrate assimilation [55]. Through denitrification, nitrite can be produced as an intermediate in the conversion of nitrate to nitric oxide, nitrous oxide and nitrogen gas [56]. Nitrite is considered harmful to fish at levels of 0.15 mg/l and above, causing conversion of haemoglobin to methaemoglobin in blood, which results in inhibition of oxygen transport and mortality due to brown blood disease [13]. Increased concentrations of nitrite also significantly affect weight gain, specific growth rate and food conversion efficiency [57].

Dietary phosphorous is an essential component of fish feeds as it improves weight gain and feed conversion ratio. It is however poorly utilized due to the absence of an acidic stomach in

some species and because phosphate is often bound to phytic acid in vegetable protein [58]. Ingested phosphorous is therefore lost in faeces and results in poor water quality with increased algal growth and eutrophication [59-60].

4. Conventional approaches for addressing challenges in aquaculture

The rearing of fish in reticulated systems results in a highly artificial environment which has a propensity for the accumulation of waste metabolites and which promotes the growth of pathogenic bacteria. Management considerations for aquaculture operations include nutrition, water quality, physical parameters and pathogen and disease control [61]. Chemicals are often used to control disease and include a wide range of topical disinfectants, organophosphates, antimicrobials and parasiticides to deal with disease and water quality [18,26]. Water quality is traditionally managed through conventional reticulated filtration systems, which are sensitive to process fluctuations and can result in mass mortality when the systems crash.

4.1. Use of chemicals in aquaculture

Antimicrobial agents are extensively used for treatment during disease outbreak or at prophylactic doses to prevent outbreak of disease. This can lead to antibiotic resistance and increased virulence of pathogenic organisms, leading to a requirement for high doses of existing drugs or new drugs to control disease [5,17,20]. Antibiotic resistance can pose a risk to human health and can cause mass mortality of fish [63]. Studies have also demonstrated that chemicals used in aquaculture can be toxic to the fish themselves, with exposure to some chemicals causing a stress response and blood biochemical changes [17,21,64]. The presence of higher drug concentrations, and an ever increasing spectrum of chemical residues, can result in detrimental effects to consumers and the environment [62]. These chemicals also have a negative impact on the aquaculture filtration systems themselves, resulting in a deterioration in water quality. Chemicals are often recalcitrant, persisting for several days to months, and can cause alterations in naturally occurring bacterial populations, Regulators have recognised the risks posed by use of chemicals as substantiated by the ever increasing list of banned substances, a consequence of which is a reduction in treatment options for aquaculture [24,65-66]. Governments and organizations have recently introduced much tighter restrictions on the use of antibiotics in animal production. As an example, the European Union (EU) banned the use of avoparcin in 1997 and in 1999 included virginiamycin, spiramycin, tylosin and bacitracin as banned growth promoters in animal feeds [67-68].

4.2. Conventional biofiltration

Normally the oxidation of ammonia to the more benign nitrate ion occurs through ammonia and nitrite oxidising obligate chemoautotrophs such as *Nitrosomonas* and *Nitrobacter spp.* which are slow growing and sensitive to fluctuations in environmental conditions [55,69]. Removal of nitrate and nitrite is a challenge in intensive aquaculture operations. System fluctuations, resulting from the sensitivity of natural filter bacteria, often lead to accumulation of ammonia,

nitrite, nitrate and phosphate. Although the concentration of these residues can be reduced by the addition of fresh water, purges of effluent containing high concentrations of these compounds into natural river and seawaters results in a deterioration of the environment and can lead to algal blooms, which may be detrimental to natural ecosystems [60]. High capital investment is thus required for installation of larger scale filtration systems to compensate for these inefficiencies of conventional filtration.

5. Biological solutions as alternatives for addressing challenges in aquaculture

Given the challenges in conventional aquaculture practise, alternative methods for disease control and enhancement of water quality are desperately required. Micro-organisms play important roles in aquaculture, particularly with respect to nutrient cycling and the nutrition of the cultured animals, water quality, disease control and the environmental impact of effluent [22]. Beneficial microbes can be used to alter or regulate the composition of bacterial flora in a water system to optimise fish production by reducing pathogen concentration, by improving water quality through reduction of waste ions and through accelerated mineralization and nitrification, by reducing algal growth and by accelerating sediment decomposition [17,20,70-71]. These biological agents also confer the added advantage of natural integration into existing ecosystems and present opportunities for development of multi-effect products which are attractive to end users. The marketing of biological and "organic certified" solutions for enhancement of fish health has also gained consumer acceptance. The use of beneficial microbes is a more appropriate remedy than the use of chemicals but successful application requires an understanding of the ecological processes occurring in aquaculture systems, of the agents responsible for disease and knowledge of the beneficial characteristics of bacteria to be used as biological agents [5,72].

5.1. Biological agents

Microbial webs are an integral part of all aquaculture systems and have a direct impact on productivity, especially in intensive culture operations. The quality of water and health of the cultured species is governed by the activities of a diversity of microbes with different roles and interactions in the ecosystem [61]. There are distinct uses of bacterial supplements in aquaculture for bio-augmentation as probiotics and as biocontrol and bioremediation agents [19]. Bio-augmentation refers to the augmentation of the environment and/or the microbes to result in enhanced fish health while probiotics are normally associated with feed and digestion. A strict definition of biocontrol agents are microorganisms that are antagonistic to pathogens. In some instances however the description of biocontrol agents transcends the boundary between bio-augmentation, and the exclusion of pathogens [73]. Bioremediation refers to the breakdown of pollutants or waste by microbes [5,61].

A probiotic can be defined as a cultured product or live microbial feed supplement which beneficially affects the host by improving its intestinal balance [74]. The important components

of this definition reflect the need for a living microorganism and application to the host as a feed supplement. A broader definition is that of a live microbial supplement, which beneficially affects the host animal by improving its microbial balance [75]. In a third proposed definition, a probiotic is any microbial preparation, or the components of microbial cells, with a beneficial effect on the health of the host [76]. It is thus apparent that there are variations in the actual application of the terminology associated with biological agents [77]. Based on the observation that organisms are capable of temporarily modifying the bacterial composition of water and sediment, it was suggested that the definition should include the addition of live naturally occurring bacteria to tanks and ponds [73]. Verschuere *et al.* [26] presented a wider and useful description, given the broad spectrum effects of microbial consortia used in aquaculture. They described a biological agent as a live microbial adjunct, which has a beneficial effect on the host by modifying the host-associated or ambient microbial community, by ensuring improved use of the feed or enhancing its nutritional value, by enhancing the host response towards disease, or by improving the quality of its ambient environment.

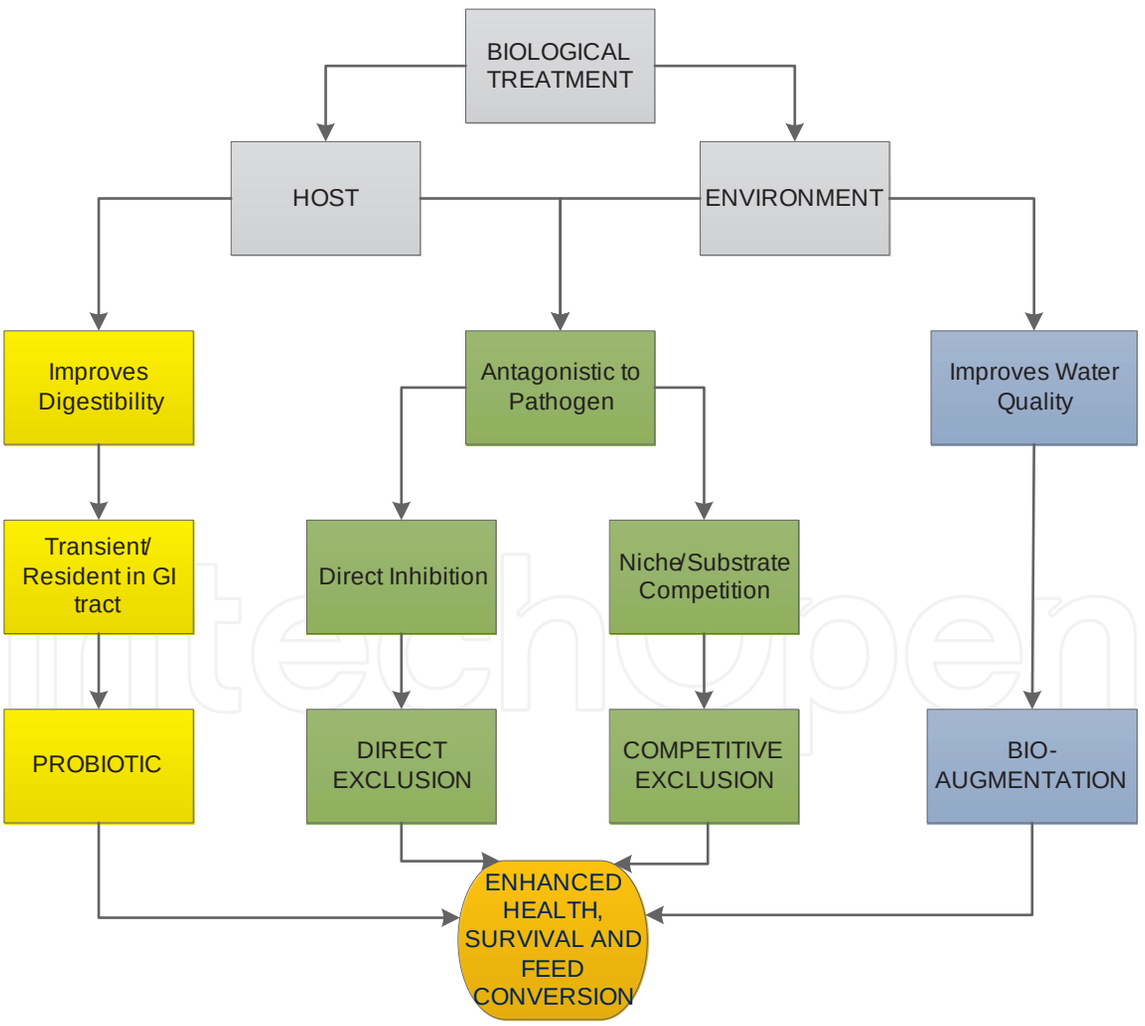


Figure 3. Schematic representation of the effects of biological agents in addressing aquaculture challenges.

The range of biological treatments examined for use in aquaculture has encompassed both Gram-negative and Gram-positive bacteria, bacteriophages, yeasts, unicellular algae, enzyme preparations and plant extracts. Microbes have been successfully applied to aquaculture systems via inclusion in artificial or live feed, by addition to biofiltration systems and by direct addition to water [77]. Most biological treatments used in aquaculture belong to the genera *Lactobacillus*, *Vibrio*, *Bacillus*, or *Pseudomonas*, although other genera have been applied to a lesser extent [26].

5.2. Modes of action of biological agents

Mechanisms of probiosis include competition with pathogens for adhesion sites, immune stimulation, synthesis of antimicrobials, competitive exclusion, bioaugmentation and bioremediation [23-24,26,78]. Although many biological treatments have been developed over the last decade, the approach used has generally been empirical and the exact modes of action were rarely elucidated, negatively affecting technology adoption and implementation in aquaculture [26].

One possible mechanism for preventing colonization by pathogens is competition for adhesion sites on gut or other tissue surfaces [78]. It is known that the ability to adhere to enteric mucus and cell wall surfaces is necessary for bacteria to become established in fish intestines [79-80]. The ability to adhere and grow on or in intestinal or external mucus has been demonstrated for fish pathogens in *in vitro* environments [81-82]. Since bacterial adhesion is important during the initial stage of pathogenic infection, competition with pathogens for adhesion receptors might be the first probiotic effect of a biological agent [81,83].

Immuno-stimulants are chemical compounds that activate the immune system of animals and render them more resistant to infections [84]. Fish larvae, shrimps, and other invertebrates have immune systems that are less well developed than their adult counterparts and are dependent primarily on non-specific immune responses for their resistance to infection [85]. Bacteria may act as immuno-stimulants in fish and shrimp, but it has not yet been conclusively demonstrated that they have a beneficial effect on the immune response of cultured aquatic species [26,86].

Microbial populations may release chemical substances that have a bacteriocidal or bacteriostatic effect on other microbial populations, which can alter inter-population relationships. The presence of bacteria producing inhibitory substances is thought to constitute a barrier against the proliferation of opportunistic pathogens. In general, the antibacterial effect of bacteria is due to the production of antibiotics, bacteriocins, siderophores, enzymes, hydrogen peroxide or alteration of pH by the production of organic acids, ammonia or diacetyl [26]. Many authors assign the inhibitory effects detected in *in vitro* antagonism tests to bacteriocins or antibiotics without investigating other possible mechanisms. It has been argued that growth inhibition could, in many cases, be accounted for by primary metabolites or simply by a decrease of the pH [26]. At this stage however, the association between amensalistic activity and *in vivo* probiotic activity is very weak and circumstantial. Typically, a correlation is made between the *in vitro* ability of the probiotics to inhibit pathogens and the *in vivo* protection of the cultured aquatic species, but in none of the studies investigated has it been shown unequivocally that

the production of inhibitory compounds is the cause of the *in vivo* probiotic activity of the strains [26]. Further research is thus required in this field.

Competition for nutrients or available energy may determine how different microbial populations coexist in the same ecosystem, but to date there have been no comprehensive studies on this subject [87]. Competitive exclusion is an ecological process that allows manipulation of the bacterial species composition in water, sediment or the host itself, by competitive assimilation of nutrients and/or an intrinsically higher growth rate [5,23-24]. The microbial ecosystem in aquaculture environments is generally dominated by heterotrophs competing for organic substrates as both carbon and energy sources. Competitive utilization of these substrates can thus attenuate target pathogenic microorganisms as demonstrated by several studies. A bacterial strain selected for its active growth in organic-poor medium, was reported to prevent the establishment of a *Vibrio alginolyticus* infection *in vivo. S*ince the strain had demonstrated no *in vitro* inhibitory effect on the pathogen it was thought to be a consequence of competitive exclusion [25]. In another example, *in vitro* antagonism tests did not show production of extracellular inhibitory compounds, yet living cells were required to protect *Artemia* against pathogenic *V. alginolyticus*. It was suggested that the selected bacteria exerted their protective action by competing with the pathogen for chemicals and available energy [26].

Virtually all microorganisms require iron for growth [88]. Siderophores are low molecular weight (< 1,500), ferric ion-specific chelating agents that can dissolve precipitated iron thus making it available for microbial growth [89]. The ecological significance of siderophores resides in their capacity to scavenge an essential nutrient from the environment and deprive competitors from accessing it. The requirement for iron is high for many pathogens in highly iron limited environments [88,90] and several studies have reported a correlation between iron availability and pathogen growth. In a challenge test with pathogenic *V. anguillarum*, salmon mortality was reported to increase linearly with dietary iron content [91]. Siderophore-producing *P. fluorescens* AH2 was demonstrated to inhibit several Gram-positive and Gram-negative bacteria, particularly when iron availability was limited [75]. *In vitro* co-culture tests revealed that the growth of *V. anguillarum* was inhibited by the filter-sterilized supernatants from iron-limited cultures of *P. fluorescens* AH2 but not from iron-replete cultures. *In vivo* studies using rainbow trout juveniles demonstrated a 46% reduction in mortality due to *V. anguillarum* infection when the culture was treated by *P. fluorescens* AH2 *in vivo*. Non-pathogenic bacteria which produce siderophores could thus be used to compete with pathogens whose pathogenicity is known to be dependent on the availability of iron [26]. It must be noted however that the body of evidence supporting the competition for free iron as a mode of action of biological agents is still scant and at present still circumstantial [26]. More recently Lalloo *et al.* [92] were able to demonstrate siderophore production as the mode of action responsible for attenuation of pathogen growth in both *in vitro* and *in vivo* studies.

Improvement in water quality has been recorded in studies involving the addition of biological agents. These improvements include the reduction of total and dissolved solids concentrations, lower concentrations of waste ions and a reduction in algal populations. Gram-positive bacteria are generally more efficient in converting organic matter to CO_2 than Gram-negative bacteria, which convert a greater percentage of organic carbon to bacterial biomass or slime.

By maintaining higher levels of these Gram-positive bacteria in production systems, farmers can reduce the build-up of dissolved and particulate organic carbon during the culture cycle [26]. Nitrite accumulation may be caused by imbalances in the activities of nitrate and nitrite reductase and inhibition of nitrite reductase by oxygen. Bio-communities however usually contain bacteria with different nitrate and nitrite reductase activities enhancing the denitrification efficiency of the overall bio-community [93]. Although the specific nitrification activity of heterotrophic bacteria is generally lower than that of chemoautotrophs, the overall impact on denitrification could be greater due to the higher cell numbers of heterotrophic bacteria and their robustness to process fluctuations. There is therefore merit in utilizing biological agents for nitrification and phosphate bioremediation to improve water quality in aquaculture [26,86]. Many bacterial strains have been shown to have a significant algaecidal effect on various species of micro algae [26,94-95]. This effect is valuable where algal blooms may be problematic, causing blockages to flow systems and changes in oxygen concentration due to algal cellular respiration.

Formulation of bacterial consortia with interactive effects, including pathogen inhibition, high growth rate and improvement in water quality, provides broad spectrum effects in a single product [72]. Lalloo *et al.* [72] obtained natural isolates from mud sediment and *Cyprinus carpio* tissue samples, which were purified and assessed in *in vitro* studies for growth inhibition of pathogenic *Aer. hydrophila* and for their ability to reduce the concentrations of ammonium, nitrate, nitrite and phosphate ions. A consortium of *Bacillus* isolates was formulated for *in vivo* trials using *C. carpio,* and demonstrated positive results for pathogen inhibition and waste ion reduction.

5.3. *Bacillus spp.* as attractive biological agents

The application of *Bacillus* species in aquaculture is growing rapidly, especially in countries where intensive systems for farming of fish and shellfish are utilised [23-24,72]. *Bacilli* are used as components of biocontrol products which are often composed of mixtures of species, which are able to exert a range of beneficial effects on aquaculture systems [24,72]. They are ubiquitous in sediments and are naturally ingested by animals [5]. An advantage of using *Bacillus spp.* is that they are not generally involved in horizontal gene transfer processes with Gram-negative organisms such as *Vibrio* and *Aeromonas spp.* and are thus unlikely to acquire genes for antibiotic resistance or virulence from these species [5]. Other key positive characteristics of this genus are the ability to replicate rapidly, tolerate a multitude of environmental conditions and provide a broad range of beneficial effects that can improve aquaculture productivity [24,27]. Additionally, the ability of *Bacilli* to sporulate enables downstream processing and formulation of shelf stable spore based products [88]. Many spore forming *Bacilli* are sold worldwide as components of products for human and animal use, including *B. coagulans*, *B. subtilis*, *B. clausii*, *B. cereus* and *B. toyoi* [23].

Several studies have demonstrated the application of *Bacillus* based products in aquaculture. *Bacillus* strain IP5832 spores fed to turbot larvae resulted in a decrease in the *Vibrionaceae* population with significant improvement in weight gain and survival of the larvae [19]. In a further study it was reported that a *Bacillus spp.* improved food absorption by enhancing

protease levels, decreased the number of pathogenic bacteria in the system and improved turbot larval growth [77]. The survival and net production of channel catfish was improved in a farm trial using a mixed culture of *Bacillus*, but the mode of action was not specified [96]. It was reported that *Penaeus monodon* larvae fed with *Bacillus* S11 fortified *Artemia* had significantly reduced development times and fewer disease problems than larvae reared in the absence of the *Bacillus* strain. When challenged with a pathogenic *V. harveyi* strain D331, survival was also significantly improved in treated groups compared to untreated controls [97]. It was also concluded, based on studies on several farms in Indonesia that the use of *Bacillus* in penaeid culture ponds enhanced the production of shrimps by preventing mortality normally caused by luminescent *Vibrio spp.* [61].

Bacillus spp. also contribute to nitrogen removal in spite of the classical belief that this process is predominated by autotrophic bacteria [55,72,93,98-102]. Some members of this group, such as *B. subtilis* and *B. cereus*, are able to grow under aerobic, facultative aerobic and anaerobic conditions, allowing for switches in nitrogen metabolism that facilitate both nitrification and denitrification [86,93,103]. The pattern of nitrite metabolism by *B. subtilis* I-41 was demonstrated as exceptional among strains which showed switching of nitrite and nitrate metabolism [55]. Nitrite oxidation might thus be common, rather than the exception, in heterotrophic bacteria such as *Bacillus spp.* [86]. The reduction of phosphate concentration in *C. carpio* culture systems has also been demonstrated through addition of *Bacillus* species [72]. The improvement in bio-availability of bound phosphate, through solubilisation, is also thought to facilitate removal of phosphate and reduce the propensity of algal blooms [60,104].

Identity of probiotic	Used on	Method of application	Reference
Bacillus sp. S11	*Penaeus monodon*	Premixed with feed	[105]
Bacillus sp. 48	*Centropomus undecimalis*	Added to water	[106]
Bacillus sp.	Penaeids	In water	[61]
B. megaterium, B. polymyxa, B. licheniformis, *B. subtilis*	Channel catfish	In water	[96]
Mixed culture, mostly *Bacillus spp.*	*Brachionus plicatilis*	Mixed with water	[107]
Bacillus spp.	*C. carpio*	In water	[72]
Bacillus strain IP5832	Turbot larvae	In water	[19]

Table 2. Summary of studies investigating the application of *Bacillus* based biological treatments.

Bacillus spp. have the ability to form endospores which are rigid structures that are capable of surviving under harsh conditions. Spores are considered metabolically inert, but can be used as biological agents due to the many advantages of this form over vegetative cells. These include their higher resistance to external factors such as mechanical force, desiccation, solar radiation and high temperatures [108]. As a consequence of this resistance to environmental

stress, spores are attractive for commercial application as they can endure harsh processing steps during production and are resilient to fluctuations in systems where they are applied, thus ensuring better survival and efficacy than vegetative cells [23]. Products containing spores can be stored in a stable form for long periods under challenging conditions normally prevalent on aquaculture farms [24,109]. *Bacillus* spores are found in the bottom of ponds, lakes and rivers and many aquatic species will naturally ingest these microbes. They generally exist in symbiotic relationships with their host [24]. Their ability to germinate selectively in response to external triggers is advantageous for application as biological agents in aquaculture, as they have the ability to recover the characteristics of a metabolically active cell in response to specific nutrients when these effects are required [108,110]. Lalloo *et al.* [88] showed that a *Bacillus* spore concentrate and powder blend were stable over a 42-day test period without significant loss in viability of spores, while final product formulations were stable for at least two years.

6. Isolation, screening and selection of candidate biological treatment agents

There is an elegant logic in isolating putative biological agents from the host or the environment in which the agents are likely to exert a beneficial effect, but there is no unequivocal indication that these isolates perform better than isolates completely alien to the cultured species or originating from a different habitat [26]. A combination of methods and incubation conditions need to be used to achieve pure cultures of target organisms. To an extent, the range of media to be used is governed by personal choice and experience [12]. Many bacteria that are residents of soil and aquatic habitats low in nutrients have difficulty growing in rich media. Also, many potential contaminants cannot compete in dilute media, so the limitation in nutrient availability becomes a selective factor. In order to appropriately select biological agents it is essential to understand the mechanisms of action and to define selection criteria for potential microbes. A classical screening and selection rationale may include collection of background information, acquisition of isolates, purification of isolates and evaluation based on pre-determined criteria for both *in vitro* and *in vivo* environments [71]. Good pre-selection criteria can include the viability of the potential probiotic within the host and/or within its culture environment, adherence to host surfaces, the ability to prevent infection by pathogenic bacteria and ability to utilise waste ions. Other selection criteria include biosafety considerations, methods of production and processing, the method of administering the probiotic and the robustness of the biological agent in the environment where the microorganisms are expected to be active. Possible pathogenicity to different life stages of the target species should also be considered. Verschuere *et al.* [26] tested their probiotics on *Artemia* to verify that the defence systems of the shrimps were able to cope with the presence of the putative probiotics.

6.1. Isolation of biological agents

When selecting desirable biological agents enrichment techniques that make it possible to exploit the differential characteristics of target isolates in mixed microbial populations

should be applied. *Bacillus spp.* are isolated almost ubiquitously from soil, water, mud, sediment, dust, air and the surfaces and organs of aquatic animals [23]. They have been isolated from fish, crustaceans, bivalves and shrimps and have been found in the microflora of the gills, skin and intestinal tract [19,24]. One effective strategy being used in developing countries is the isolation of *Bacillus spp.* from commercial ponds and then using selected isolates as commercial products [24]. *Bacilli* are classified as endospore forming Gram-positive rods and cocci and isolation procedures must selectively enrich for these organisms while excluding other genera in the same group. In one study, methods used for isolating various *Bacillus* strains were based mainly on the resistance of their endospores to elevated temperatures [111]. The technique used involved blending of samples with an enrichment medium, which also induced vegetative cells to sporulate, followed by incubation to allow formation of mature spores in large quantities. The isolation involved heat treatment for the selection of spores from *Bacillus* species. Ethanol is also a useful disinfectant and dehydration agent to use for isolation of *Bacillus* strains as its application kills vegetative cells, whereas the more resistant endospores survive. The resistance of *Bacilli* to the antibiotic polymyxin B also enables use of this antibiotic for the selection of this group of bacteria whilst eliminating most Gram-negative bacteria. Once selected, cells can be characterised by their morphology, typically using microscopic techniques, by use of gram staining and by quantification of the activity of enzymes such as catalase [111].

6.2. *In vitro* screening and selection of aquaculture biological agents

To appropriately select biological agents it is essential to understand the mechanisms of action and to define selection criteria for potential probiotics [112]. Many bacteria have been exploited as biological agents but their selection has been based mainly on empirical observations rather than scientific data [71].

A common protocol for screening candidate biological agents is to perform *in vitro* antagonism tests, in which pathogens are exposed to antagonists in culture medium [75,80,113-116]. Assays for the production of inhibitory compounds and siderophores, or the competition for nutrients, are some common strategies that have also been used [75,80,117-119]. Results of *in vitro* antagonism tests should however be interpreted with caution as growth media and conditions can influence the effects observed which may differ from the actual activity *in vivo* [80,120]. The pre-selection of candidate probionts based on *in vitro* antagonism tests has however led to the discovery of many effective probionts and is a useful first step in selection [116]. The use of the target organism in the screening procedures provides a stronger basis for selection of antagonists [26]. The target species should be challenged under normal or stress conditions with the candidate biological agent. Growth inhibition may not always be a consequence of the production of inhibitory substances such as antibiotics, as inhibition caused by other mechanisms must also be considered during *in vitro* screening tests [121-122]. As an example Lalloo *et al.* [88] investigated the mode of action of a novel *B. cereus* isolate for the inhibition of pathogenic *Aer. hydrophila*. The production of antimicrobial compounds was excluded as the mode of action based on the absence of growth inhibition of *Aer. hydrophila* by intracellular or extracellular fractions of *B. cereus.* In contrast, actively growing *B. cereus* cells inhibited the

growth of the *Aer. hydrophila*. Based on co-culture data, competitive exclusion through an intrinsically higher growth rate and competitive uptake of essential nutrients was identified as the mode of action. Co-cultivation of *B. cereus* with *Aer. hydrophila* resulted in a 70% reduction in the cell density of the pathogenic organism in a remarkably short time period. These findings confirmed previous work where a decline in pathogen levels was demonstrated in both *in vitro* and *in vivo* studies when *B. cereus* was administered as a biological agent [72]. Further studies investigated the effect of iron availability on pathogen growth and demonstrated the superior efficiency of *B. cereus* in assimilating iron, resulting in a decline in pathogen levels in iron deficient medium. [88].

In aquaculture, bioremediation or bioaugmentation is an important selection criterion, particularly under conditions that mimic the application environment [5]. While some studies have reported screening strategies to select for the bioremediation capabilities of potential aquaculture biological agents, this area has regrettably not been well reported to date [27,72]. Recent studies by Lalloo *et al.* [72] described methodology applied for the selection of *Bacillus spp*. based on their ability to utilise ammonia, nitrate, nitrite and phosphate ions.

Once candidate biological agents are selected, proper identification and safety assessment is an important requirement prior to application *in vivo*. Identification can be performed using techniques such as 16S RNA sequence homology. Where close sequence homology is found between species of potentially dangerous genera, additional assessment may be necessary. Lalloo *et al.* [72] demonstrated that their *B. cereus* isolate was negative for anthrax toxins and did not contain the anthrax virulence plasmids pXO1, pXO2 or the *B. cereus* enterotoxin. These studies were necessitated by the high sequence homology found between *Bacillus* species. Toxicity towards the cultured species can also be employed in screening strategies. As an example, Austin and Austin [12] tested their candidate biological agent by injection into Atlantic salmon followed by histopathological examination of the kidneys, spleen and muscles.

6.3. *In vivo* validation of the efficacy of putative biological agents

Once candidate biological agents have been selected, the next important step is confirmation of observed efficacy using *in vivo* tests. The use of small scale model *in vivo* systems is a cost effective method that allows more certainty in selection of candidate biological agents [26,94]. These tests may measure various effects, including antagonism, by including an experimental infection with a representative pathogen. Pathogens can be administered via the diet, through immersion, by injection or via the culture water [123]. To determine the effects of a specific bacterial strain on a cultured organism, the elimination of other microbes from the culture system may be necessary [124]. This approach can also be used to examine other effects on water quality and the impact on other trophic levels, such as algae [94-95,125]. With *in vivo* challenge tests, changes in population dynamics of the antagonist and the pathogen as well as other effects on the culture system should be studied. Of importance are the unintended negative effects on the target species and interference with filtration efficiency in reticulated culture systems [26,123]. As an example, in an oyster culture system, a decrease in the level of the pathogen *V. tubiashii* was observed when an *Aeromonas* probiotic strain was added together

with the growth media of the probiotic strain. The putative antagonist itself could not however be detected in the culture after four days. This example shows the importance of measuring interactions, including mortality or disease, after experimental infection and to include appropriate controls in study designs [116]. In another study, the efficacy of a *B. cereus* isolate was demonstrated *in vivo* based on predefined criteria. In addition to this, the tolerance to, and functionality across, a range of physiological conditions in systems used to rear *C. carpio* was also proven [27,92]. Furthermore, the *in vivo* treatment did not result in a negative impact on oxygen sufficiency, growth or health of the specimens, which are all important considerations for application of biological agents [21].

6.4. Other considerations during selection of biological agents

Strains showing well-established biological effects in *in vitro* and *in vivo* studies need to be tested for their suitability to real world biological treatment applications. Additional criteria such as biosafety considerations, methods of production and processing, the method of administering the probiotic and the environmental conditions where the microorganisms are expected to be active are important considerations [112]. An isolate cannot be used as a probiotic unless it has been confirmed as non-pathogenic to the host, to humans and to the environment [26]. Relevant legislation, if any, should be taken into account before commencement of commercial application. Finally, a cost-benefit analysis will determine whether the probiotics can be applied in practice or not [26].

7. Bio-production of biological agents

Large scale production of probiotics is an essential step towards application in the aquaculture industry as production cost is an important consideration in the development of commercially relevant biological products [126]. The cultivation of microorganisms at a large scale is influenced by various factors such as the composition of the media, as well as physical and chemical variables [127]. It has been widely documented that nutrient sources influence the growth, spore production, and synthesis of commercially useful metabolites in *Bacillus spp.* [128-130]. The nutritional and physicochemical parameters of the fermentation process thus need to be optimized, with use of economical and commercially available media a key consideration to reduce costs of bio-production [131-132]. Media formulation and optimization are key considerations for the production of affordable aquaculture biological agents, yet limited progress has been made in this area to satisfy market opportunities for affordable commercial aquaculture products [126,77]. With increased cell yield, productivity and cost reduction, the fermentation production process can be made feasible and economically attractive for application of aquaculture products [128]. Another key consideration is that scale up of production must not compromise product efficacy or amenability to stabilization and formulation [133]. Genetic engineering provides an option for improvement of biological agents; however public resistance to genetically modified organisms, particularly when associated with food production is an important consideration before adopting this approach.

7.1. High cell density cultivation of *Bacillus spp*.

Although *Bacillus* biological agents are widely used in aquaculture, there are limited studies on their production and little is known about the impact of nutrient supplementation on high-density production of bacterial spores by fermentation [134-135]. Carbon and nitrogen sources generally play a dominant role in the productivity of a fermentation process as these nutrients are directly linked with the production of the biological agent [136-137]. According to current understanding, the development pathway leading from a vegetative cell to a spore is triggered by depletion of carbon, nitrogen, phosphate or essential micronutrients [138-140]. A suitable medium must thus support vegetative growth and also the production of spores [141]. It has been widely documented that nutrient sources influence the growth, spore production and synthesis of commercially useful metabolites in this species [128-130].

The type of carbon source and the carbon to nitrogen ratio play an important role in microbial growth [142]. It has been observed that *B. subtilis* uses glucose as its major carbon source and the efficiency of carbon utilisation towards biomass formation is low when the glucose concentration exceeds ~10 g/l in batch culture [143]. The production of by-products is increased in the presence of excess glucose, resulting in reduced yields of biomass, which is undesirable when producing biological agents [143]. Certain over-flow metabolites can also inhibit cell growth [143]. Monteiro *et al.* [134] also observed that an increase in glucose concentration up to 5 g/l led to an increase in the vegetative cell and spore concentration of *B. subtilis*, while higher sugar concentrations inhibited sporulation. It is therefore of great importance to regulate carbon availability to optimise growth and sporulation parameters precisely [127]. It is also noteworthy that the glucose consumption rate depends significantly on factors such as pH and oxygen sufficiency. Mass transfer parameters such as agitation and aeration are thus important in maximising vegetative cell growth, without inducing a premature onset of sporulation [144]. In most studies, glucose was found to be the best carbon substrate for the production of *Bacillus spp.* and their spores [145].

Various protein substrates have been tested for the growth and synthesis of commercially useful metabolites by *Bacillus spp.* [27,128-130,146]. It has furthermore been widely documented that protein sources influence spore production in this species [141,147-149]. Commonly used nitrogen based nutrient sources include a wide range of peptones, extracts and hydrolysates, many of which are too expensive for industrial scale manufacture of large volume products and have negative market acceptance if they contain animal by-products [143,150]. Media formulated to support high productivities are thus predominantly formulated with inexpensive complex nitrogen sources [137,151]. Although yeast extract, peptones and meat extracts have been shown to improve bacterial growth rate as they are good sources of protein, vitamins and co-factors, there have been reports suggesting that metabolite production, and particularly spore production, are often better when corn steep liquor (CSL) is used [128-129, 135,141,146,152-153]. CSL contains a wide range of macro and micro elements known to be important for spore production [154]. Lalloo *et al.* [27] demonstrated an attractive material cost of production at the optimal supplementation level for CSL, reducing the overall production cost by using this inexpensive source of nitrogen. They also demonstrated that CSL was a preferred nutrient substrate for the production of *Bacillus* spores, in comparison to conven-

tional nutrient substrates. The use of CSL resulted in a higher spore concentration, productivity and spore yield on protein, in comparison to yeast extract and nutrient broth. Apart from the nature of protein source, the protein concentration in culture media also affects growth and spore production [155]. *B. subtilis* spore productivity increased, but spore yield decreased, with an increase in CSL concentration [149]. The yield of spores on carbohydrate increased with increasing concentration of CSL, suggesting that a higher protein to carbon ratio was preferable for production of *B. subtilis* and *B. licheniformis* spores [143,156]. High levels of CSL supplementation (~60 g/l) however resulted in slow growth, cell lysis and poor spore formation as sporulation efficiency is known to be low following poor growth [141,157-158]. Sporulation takes longer in high cell density cultivations, thus resulting in a compromise between spore concentration and productivity [138]. A major advantage of CSL is that it is available in an ultra-filtered phytase treated variant, which is cost competitive and offers processing advantages in both up-stream and down-stream process unit operations [88,156,158]. Precipitation and mass transfer issues are reduced when using this form of CSL for high cell density cultivation, due to hydrolysis of phytic acid and the removal of solids through the ultra-filtration process [134,138]. As this CSL is not spray dried, as is typical of the conventional type, degradation of vitamins and key nutrients is reduced which improves growth performance [129]. Of peripheral benefit is the use of a corn wet processing waste which improves value addition and reduces environmental pollution normally caused by such materials [128].

7.2. Production of spores

The key challenge in spore production is to maximize sporulation from a high density vegetative cell culture [134,139]. Environmental signals for sporulation include culture density dependant peptides, oxygen availability and limitation of carbon, nitrogen or phosphorous [140]. The life-cycle of a spore forming bacteria consists of four stages i.e. vegetative growth, sporulation, germination and outgrowth [139,159]. Cells enter a sporulation pathway, which involves three differentiating cell types, namely the predivisional cell, mother cell and the forespore, in response to nutrient limitation [160]. The forespore undergoes dehydration, while the cortex is produced between the two membranes that separate the mother cell and the forespore. Eventually the mature spore is released when the mother cell lyses. This mature spore has the ability to remain dormant for long periods of time [160]. The most important sporulation related transcriptional regulator is Spo0A which is phosphorylated via a complex network of interactions in response to nutrient limitation [140,161]. Furthermore, genes in the Res system are induced under anaerobic growth conditions which contribute to the sporulation cascade during oxygen insufficiency [162]. Low phosphate concentration results in the earlier onset of sporulation due to the response of the Pho system to phosphate starvation [162]. Magnesium sulphate, calcium carbonate and phosphate all stimulate sporulation, whereas divalent cations (particularly Ca^{2+}) assist in dehydration and mineralization of the spore [154,161]. According to Monteiro *et al.* [134], the sporulation efficiency for *B. subtilis* was found to be independent of the pH values within the range of 6.9-9.0. For several *Bacillus spp.* sporulation is highly related to O_2 supply and it has been reported that non-limited oxygen conditions during the growth phase are important to realise high spore yields [163-164].

8. Application of biological agents

A key challenge for usefulness of biological agents is the survival of the micro-organisms in the environment to which they are applied. Biological agents must thus be tolerant to the prevailing environmental conditions in which they are expected to perform, often dictated by the species being cultured for a specific aquaculture application [49]. Several methods of addition of biological agents to the host or its ambient environment exist, with each application method presenting unique challenges to the survival and efficacy of the biological agent [26,71,118,165]. A biological agent must provide actual benefit to the host, be able to survive in the environment of the intended application and should be stable and viable for prolonged storage and in the field [77]. Other factors such as natural deterioration and washout of the biological agent may necessitate the on-going addition of the treatments to maintain their positive effect [26]. Information on the robustness and functionality of biological agents in response to environmental conditions such as salinity, pH and temperature are however limited. Lalloo *et al.* [92] demonstrated that temperature had a significant influence on germination, specific growth rate and increase in cell number of *B. cereus* in shake-flask cultures, whilst salinity and pH did not have a measurable effect on growth. Changes in the above conditions influence spore germination, cell growth, survival and the functionality of *Bacillus spp.* as aquaculture biological agents [166]. A key consideration for the application of *Bacillus* based biological agents is that the spores need to germinate and grow, such that the characteristics of a metabolically active cell can be recovered [110]. The replication of vegetative cells can further enhance the bioactivity in the intended application. Spores lose their dormant properties when conditions are favourable in the presence of specific germinants such as nutrients [167]. However, the germinant has to penetrate the outer coat and cortex layers of the spore before coming into contact with specific germinant receptors [110,168]. The germination of spores is a sensitive transition state involving the initiation of metabolism [169].

For the application of spores as aquaculture biological agents, determination of their functionality as antagonists to disease or for improvement in water quality under the physiological ranges to be encountered in the aquaculture system is thus an important requisite [88]. Changes in growth conditions such as temperature constitute a key factor that influences cell growth and survival of *Bacillus spp.* in their habitats. *B. subtilis* has the ability to sustain growth across a wide temperature range from approximately 11ºC to 52ºC [139,170]. When the growth temperature for *B. subtilis* is increased rapidly, changes in gene expression occur, which is known as a heat shock response. A cold shock response is elicited when the temperature is dropped down to 15ºC from 37ºC [166]. *B. cereus* is apparently not well adapted to cold temperatures and the metabolic rate decreases drastically below 13ºC [171]. A useful method for the elucidation of temperature domains for prediction of functionality of a biological agent is by examining conformance of efficacy measures to Arrhenius and Ratkowsky functions [171-172]. The vegetative cells of *B. cereus* are more sensitive to acidic conditions than spores. However, like many other cells, vegetative cells of *B. cereus* have the ability to induce an acid tolerance response [173]. The mechanisms of resistance to acidic conditions involve three factors i.e. (i) F_0F_1 ATPase and glutamate decarboxylase (ii) metabolic modifications and (iii) protein synthesis to protect and repair macromolecules [173]. *B. cereus* spores were shown to

be tolerant to the salinity and pH extremes typically encountered in the culture of ornamental *C. carpio* [174-175]. It is noteworthy that the efficacy of biological agents to environmental conditions must be assessed in line with the dynamics of the target species and the aquaculture system in response to these physiological ranges. As an example, reduced activity of a biological agent at lower temperature does not necessarily indicate a failure of the biological agent to perform, as lower temperature could translate to a lower intake of feed, waste metabolite generation and pathogen propensity in the aquaculture system.

9. Future prospects of the technology

The traditional practise of extensive land based aquaculture is under pressure, due to a limitation in available space, which has led to the increased use of more intensive reticulated systems which also offer the benefit of greater control of physiological culture conditions. While intensive systems offer the advantages of increased stocking densities and higher production throughput, challenges include water quality and increased disease prevalence among others. These are driving the adoption of environmentally friendly solutions that meet consumer expectations and comply with regulatory requirements. Biological solutions provide an attractive option. Issues that require attention to accelerate the adoption of biological solutions include the elucidation of the mode of action of commercial biological products and demonstration of clear cost-benefit advantages for commercial products.

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