Competitive exclusion as a mode of action of a novel *Bacillus cereus* aquaculture biological agent

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ABSTRACT

Aims: To determine the contribution of potential modes of action of a *Bacillus cereus* aquaculture biological control agent in inhibition of the fish pathogen, *Aeromonas hydrophila*.

Methods and Results: When *B. cereus* was tested in plate well inhibition studies, no production of antimicrobial compounds was detected. *B. cereus* had a high growth rate ($0.96.h^{-1}$), whereas *Aer. hydrophila* concentration decreased by ~70% in co-culture experiments. In nutrient limitation studies, *B. cereus* had a significantly higher growth rate when cultured under glucose ($p<0.05$) and iron ($p<0.01$) limitation in comparison to *Aer. hydrophila.* *B. cereus* glucose (0.30 $g.l^{-1}.h^{-1}$) and iron (0.60 $mg.l^{-1}.h^{-1}$) uptake rates were also significantly higher ($p<0.01$) than the *Aer. hydrophila* glucose (0.14 $g.l^{-1}.h^{-1}$) and iron (0.43 $mg.l^{-1}.h^{-1}$) uptake rates. Iron uptake was facilitated by siderophore production shown in time profile studies where relative siderophore production was ~60% through the late exponential and sporulation phases.

Conclusions: Competitive exclusion by higher growth rate, competition for organic carbon and iron, facilitated by siderophore production, could be identified as mechanisms of pathogen growth inhibition by *B. cereus.*

Significance and Impact of the Study: This study is the first elucidation of the mechanism of action of our novel *B. cereus* biological agent in growth attenuation of pathogenic *Aer. hydrophila*. This study enhances the application knowledge and attractiveness for adoption of *B. cereus* NRRL 100132 for exploitation in aquaculture.

Keywords

Bacillus spp., biological agent, aquaculture, mode of action, siderophores

INTRODUCTION

Global aquaculture is challenged by poor water quality and the outbreak of diseases (Jeney and Jeney 1995; Moriarty 1999). The use of conventional chemotherapies has resulted in the increased virulence of pathogenic strains, negative environmental impact and is often met with consumer resistance (Vershuere, et al. 2000). Exploitation of beneficial bacteria as biological agents has potential advantages to address aquaculture challenges, by improving water quality and reducing disease propensity caused by pathogenic bacteria (Fast and Menasveta 2000; Gomez-Gill et al. 2000; Jana and Jana 2003; Hong et al. 2005). Water quality and infection by pathogenic *Aer. hydrophila* are major challenges in the highly lucrative aquaculture of *Cyprinus carpio*.

A novel *Bacillus cereus* (NRRL100132) strain was previously isolated as a biological agent for *C. carpio* and its outstanding capability in enhancing water quality and reducing *Aer. hydrophila* growth was demonstrated in both *in-vitro* and *in-vivo* studies (Lalloo et al. 2007). This *B. cereus* is a water additive and was shown to be safe for use. The functionality of the micro-organism was also demonstrated across a range of physiological conditions, prevalent in aquaculture (Lalloo et al. 2008). Spore-forming *Bacillus* spp. are attractive as biological control agents as they possess antagonistic effects on pathogens, can improve water quality and are ubiquitous in natural environments (Hong et al. 2005; Wolken et al. 2003). Spores are physiologically robust and can be formulated into stable commercial products which are tolerant to the environmental conditions required in their application (Gross, 2003; Lalloo et al. 2009).

The success of strategies using biological agents and adoption of this technology by the aquaculture industry depends on an understanding of the beneficial characteristics and mechanism of action (Vershuere et al. 2000; Vine et al. 2006). However, studies showing the mode of action for antagonism of *Aer. hydrophila* by *Bacillus* spp. are limited, while no studies on the mode of action of *B. cereus* as a biological agent against this pathogen have been reported (Kumar et al. 2006; Newaj-Fyzul et al. 2007). Potential mechanisms of biological agents against pathogens include competition for adhesion sites, production of enzymes, immune stimulation, synthesis of antimicrobials, competitive exclusion and bioremediation (Hong, 2004; Vanbelle, 1990; Sanders, 2003; Verschuere et al. 2000). The basis of competitive exclusion is through competition for chemicals or for available energy or by intrinsic growth rate advantage (Vershuere, et al. 2000, Holzapfel et al. 2001, Irianto and Austin, 2002, Hong et al. 2005). Many of these mechanisms only apply to probiotics added to feed, but the latter three are relevant to water borne additives such as *B. cereus*.

The bioremediation capability for ammonium, nitrite, nitrate and phosphate waste removal by *B. cereus* NRRL 100132 was well elucidated previously (Lalloo et al. 2007). Likely modes of action by our *B. cereus* isolate in antagonism of *Aer. hydrophila* are the production of inhibitory compounds and competitive exclusion. Fastidious heterotrophs such as *Bacillus* spp. often demonstrate a high utilization of organic carbon (Verschuere et al. 2000). Some are also capable of synthesizing low-molecular weight chelating compounds called siderophores which facilitate competitive uptake of iron for growth (Vershuere, et al. 2000; Winkelmann 2002). As both carbon and iron are essential requirements for growth by most organisms, limitations can result

in growth attenuation (Braun et al. 1999). In this study, we investigated the contribution of direct inhibition by production of extracellular inhibitory compounds and competitive exclusion through growth rate advantage, competition for key nutrients such as organic carbon and iron as potential modes of action involved in the inhibition of the important fish pathogen, Aer. *hydrophila* by our novel *B. cereus* aquaculture biological agent.

MATERIALS AND METHODS

Detection of antimicrobial activity of *B. cereus* NRRL 100132

The production of antimicrobial compounds by *B. cereus* NRRL 100132 was assessed by culturing the strain in 2L Braun Biostat B fermenters (Sartorius BBI Systems, Melsungen, Germany) as previously described (Lalloo et al. 2009). Airflow was maintained at 1 $v.v^{-1}.m^{-1}$ and agitation speed was ramped from 500rpm to a maximum of 1000rpm to maintain oxygen saturation above 30%. All materials used in this study were obtained from Merck (Darmstadt, Germany) unless otherwise stated.

Fermenters were sampled during early exponential, mid exponential and the sporulation phase. The growing culture (fermentation broth sample), intracellular cell fraction and extracellular supernatant were evaluated for the presence of inhibitory compounds. The extracellular fraction was the resultant supernatant after centrifugation of the whole broth at 13000 x g. The resultant cell pellet was washed, re-suspended in saline (0.9% $m.v^{-1}$ NaCl) and ultra-sonicated at a frequency of 20 $kHz.s^{-1}$ at 192 watts on ice for 12 min (12 x 48s cycles of sonication with a 12s pause between cycles) and then re-centrifuged. The supernatant of this cell preparation was used

as the intracellular fraction. Cell preparations (100µl) of growing culture, intracellular fraction or extracellular supernatants were loaded into wells (10mm) on nutrient agar plates pre-spread with *Aer. hydrophila* (ATCC 7966) culture. Plates were incubated (12h, 32^{o}C) and visualized for zones of inhibition.

Co-culture of *B. cereus* and *Aer. hydrophila* in shake flasks

Stored cryo-cultures (2ml) of *Aer. hydrophila* and *B. cereus*, prepared according to Meza et al. (2004), were used to inoculate triplicate 1l Erlenmeyer flasks, containing Synthetic Pond Water (SPW) growth medium and the culture flasks incubated (Lalloo et al. 2007). Samples were taken two hourly and cell counts were performed using a Thoma® bacterial counting chamber (Hawksley & Sons, London, England) for both organisms.

Comparison of growth rate between *B. cereus* and *Aer. hydrophila* under nutrient limitation

The impact of nutrient limitation on growth of *B. cereus or Aer. hydrophila* was assessed by lowering the concentration of one media component (glucose, nitrite, nitrate, ammonia, iron or phosphate) in SPW to 10% of base case. De-ionized water was the negative control and SPW was the positive control.

Media were prepared by combining amino acid, vitamin, trace element, nutrient and ion solutions. Each media formulation contained 20µl of an amino acid solution (45mg.l^{-1} each of the following: alanine, arginine, aspartic acid, glutamic acid, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine and valine), 20µl of a vitamin solution

(Lalloo *et al*., 2009) and 20µl of a trace element solution ($CaCl_2$ 3.4 mg.l^{-1}, $MgCl_2.4H_2O$ 2.6 mg.l^{-1}, H_3BO_3 5.0 mg.l^{-1}, $Na2MoO_4.2H_2O$ 0.3 mg.l^{-1}, $CoCl_2.6H_2O$ 0.4 mg.l^{-1}). The nutrient solution (glucose 10.0g.l^{-1}) and ion solution ($NaNO_2$ 0.6 g.l^{-1}, KNO_3 0.85 g.l^{-1}, $FeC_6H_6O_7$ 0.16 g.l^{-1}, $(NH_4)_2SO_4$ 0.93 g.l^{-1} and H_3PO_4 3.8 g.l^{-1}) were added as 20µl aliquots to the media. Once all media components were added the volume of each well was made up to 200µl with de-ionized water. All solutions were sterilized by filtration through 0.22µm filters.

Cultures of *B. cereus* NRRL 100132 or *Aer. hydrophila* (ATCC 7966) were grown (Lalloo et al. 2007) to 1×10^5 cells.ml^{-1} and an inoculum volume of 10µl was used to inoculate the respective micro titre wells (six wells per organism per test). Plates were incubated at 32ºC for 24 hours on a microtitre plate shaker set at 100rpm and absorbance was measured and recorded every hour at 660nm (Abs_{660}) using a BioTek Power wave^{HT} microtitre plate reader (BioTek Instruments Inc, USA). Growth rates were determined from plots of the natural logarithm of Abs_{660} over time, conforming to linearity ($r^2>0.9$). The growth rates obtained for both *B. cereus* and *Aer. hydrophila* were compared (ANOVA) to assess the impact of the individual component limitations on the growth of the two organisms (Table 1).

Measurement of glucose and iron uptake rates

Cryopreserved cultures of *B. cereus* or *Aer. hydrophila* were used to inoculate 1L Erlenmeyer flasks containing 100ml of sterile SPW in triplicate and incubated as previously described. Samples were taken on an hourly basis and analysed for iron and glucose concentrations. Iron concentrations were determined using a Spectroquant® kit 1.14549.0001 (Merck, Darmstadt, Germany). Glucose concentrations were determined using an HPIC (CarboPac^{TM}, PA1 column,

Dionex, MA, USA). Uptake rates were calculated from plots of concentration of iron or glucose against time for each microorganism.

Measurement of siderophore production

B. cereus (NRRL 100132) was used to inoculate 100ml of sterile SPW in 1L Erlenmeyer flasks and incubated as previously described. Flasks were sampled two hourly, and the cell and spore concentrations were determined, from which the sporulation ratio was calculated (Monteiro et al. 2005). Qualitative siderophore production using a modified chrome azurol S assay (Milagres et al. 1999) and semi-quantitative siderophore production using the CAS universal siderophore assay (Schwyn and Neilands, 1986) were assessed. The qualitative assessment of siderophore production in the culture medium was visualized by a colour change from blue to orange on modified CAS-agar plates. For the semi-quantitative assay, the amount of siderophore present in the test sample was reported as a percentage relative to a control sample of which the siderophore concentration was known.

RESULTS

Inhibition of growth by production of an antibacterial compound

Zones of inhibition of *Aer. hydrophila* growth were observed during the exponential, early stationery and sporulation phases when viable cells were tested in plate well assays. . However, plate well assays testing intracellular extracts or extracellular supernatants, did not show any antagonism of *Aer. hydrophila* by *B. cereus* during the entire growth cycle (Table 1).

Investigation of competitive exclusion in co-culture studies

Co-culture experiments were conducted by cultivating *B. cereus* and *Aer. hydrophila* together in shake flasks. *B. cereus* displayed a typical growth profile (μ =0.96), but there was a drastic decrease in the cell density of the pathogenic *Aer. hydrophila* population. When *B. cereus* cell concentration peaked, the pathogen had decreased by more than 70% of the starting concentration (Figure 1).

Effect of individual nutrient components on antagonism against the pathogen measured by differential growth rates

B. cereus had a significantly higher growth rate in comparison to *Aer. hydrophila* when cultivated in SPW as a positive control (p=0.003), SPW with low iron concentration (p<0.001), and SPW with low glucose concentration (p<0.05) (Table 2). When media contained reduced concentrations of ammonia, nitrite or nitrate, there was no significant difference in growth between the two organisms (p > 0.05) (Table 2). Neither of the microorganisms grew in treatments where phosphate was limited (Table 2).

Evaluation of iron and glucose uptake rates by *B. cereus* and *Aer. hydrophila*

During separate batch cultivations under identical conditions, *B. cereus* and *Aer. hydrophila* demonstrated classical exponential growth curves. Trends for glucose uptake from the growth media were linear (r^2>0.9), but not for iron uptake by either of the micro-organisms (Figure 2). *B. cereus* had an overall iron uptake rate of 0.60 $mg.l^{-1}.h^{-1}$ and a glucose uptake rate of 0.30 $g.l^{-1}.h^{-1}$. These uptake rates were significantly higher (p<0.01), than that of *Aer. hydrophila* for iron (0.43 $mg.l^{-1}.h^{-1}$) and glucose (0.14 $g.l^{-1}.h^{-1}$), respectively.

Evaluation of the production of siderophores

In the qualitative siderophore plate assay, *B. cereus* colony-forming units with orange halos were observed during the exponential growth and sporulation phases (data not shown). This observation was confirmed in the *B. cereus* culture study, where siderophore production was assessed. A maximum growth rate of 0.7 h^{-1} and cell concentration of ~7.00×10^7 cells.ml^{-1} was achieved (Figure 3a). The culture reached a high sporulation ratio at ~12 hours of growth (Figure 3b). There was a gradual increase in the production of siderophores during the course of the cultivation (Figure 3c), reaching a maximum relative sideropohore production of 65% as the culture entered the stationary phase. After completion of sporulation, the siderophore concentration remained at a constant high level.

DISCUSSION

The mode of action of a novel *B. cereus* isolate as a biological agent in aquaculture for the inhibition of pathogenic *Aer. hydrophila* was investigated. The production of antimicrobial compounds by *B. cereus* was excluded as a mode of action, based on the absence of growth inhibition of pathogenic *Aer. hydrophila* by intracellular or extracellular fractions of *B. cereus* (Table 1). In contrast, actively growing *B. cereus* cells caused growth inhibition of *Aer. hydrophila.* Although production of antimicrobial compounds is a common mode of action exploited for attenuation of a selected target pathogen in an environment (Fredrickson and Stephanopoulos, 1981; Hong et al. 2005), this mechanism did not apply to *B. cereus* NRRL100132. Similar to our findings, Brunt and Austin (2005) showed that their *Bacillus* obtained from the digestive tract of carp, also inhibited the growth of pathogenic *Lactococcus*

garvieae and *Streptococcus iniae* without showing any signs of antibiosis, thus indicating an alternate mode of action other than production of antimicrobial compounds.

Competitive exclusion through an intrinsically higher growth rate and competitive uptake of essential nutrients was identified as a mode of action involved in the antagonism of *Aer. hydrophila* by *B. cereus*, based on co-culture data (Figure 1). Co-cultivation of *B. cereus* together with *A. hydrophila* in SPW resulted in a decline of more than 70% in the cell density of the pathogenic organisms in a remarkably short time period (Figure 1).

Competitive exclusion was partly attributed to a substantially higher growth rate of *B. cereus* ($0.96h^{-1}$) in comparison to *Aer. hydrophila,* where cell death was observed. These findings further confirmed our previous work where pathogen decline was proven in *in-vitro* and *in-vivo* studies, when *B. cereus* was administered as a biological agent (Lalloo et al. 2007). Several previous studies have reported higher growth rate as a likely mechanism of biological agents in the inhibition of other microorganisms (Moriarty, 1998; Pinchuk et al. 2001; Patterson and Burkholder, 2003).

In addition to the intrinsically higher growth rate, competition for the essential nutrients, glucose and iron, contributed to the mechanism of competitive exclusion of *Aer. hydrophila* by *B. cereus*. Competitive exclusion by an intrinsically higher growth rate is often linked to competitive uptake of essential nutrients such as iron and glucose (Rico-Mora et al., 1998; Vershuere et al., 2000). As *B. cereus* and *Aer. hydrophila* are both heterotrophic, competition for organic substrates as both carbon and energy sources could be expected, although this mode of action for

the inhibition of *Aer. hydrophila* by *B. cereus* has not been demonstrated previously (Vershuere et al. 2000). In nutrient limitation studies, *B. cereus* had a significantly higher growth rate than *Aer. hydrophila* in both SPW and SPW with limited glucose or iron (Table 2). We further confirmed these observations in glucose and iron uptake studies (Figure 2), which indicated a significantly higher uptake ($p<0.001$) of glucose (0.30 $g.l^{-1}.h^{-1}$) and iron (0.60 $mg.l^{-1}.h^{-1}$) by *B. cereus* in comparison to *Aer. hydrophila* for glucose (0.14 $g.l^{-1}.h^{-1}$) and iron (0.43 $mg.l^{-1}.h^{-1}$) respectively. When *Aer. hydrophila* iron uptake rates were evaluated, a four hour lag was observed, whereas *B. cereus* uptake was immediate (Figure 2). These results indicated that competition through higher growth coupled to the competitive uptake of glucose and iron were key modes of action for antagonism by *B. cereus* (Veshuere et al. 2000; Patel et al. 2009).

The mechanism of competitive exclusion by competition for iron uptake was facilitated by siderophore production by the *B. cereus* isolate. The strain of *B. cereus* exhibited a growth associated increase in siderophore concentration during the exponential phase of growth (Figure 3c). Most importantly, the siderophores remained in the medium during and post-sporulation. These results correlated with the work conducted by Patel et al. (2009), where siderophore production increased during the exponential phase of growth and remained stable during the sporulation phase, with a similar level of siderophore production to the present *B. cereus* isolate. A qualitative assay revealed a large number of colony forming units with orange halos (data not shown), confirming the presence of siderophores (Milagres et al. 1999). Prior research conducted by Park et al. (2005) and Wilson et al. (2006) also specifically demonstrated the ability of *B. cereus* to produce siderophores. Studies carried out by Gram et al. (1999) and Smith and Davey (1993), demonstrated a positive correlation between the production of siderophores and a

decrease in pathogen prevalence. Although *Aer. hydrophila* is itself capable of synthesizing low-molecular weight siderophores, termed 'amonabactins', production of the siderophore is thought to be inducible and regulated by extracellular iron concentration (Chart & Trust, 1983). *B. cereus* was able to produce siderophores immediately at the start of batch culture (Figure 3), thereby decreasing the iron concentration to very low levels within the first five hours (Figure 2) thus starving *Aer. hydrophila* of iron.

The modes of action for attenuation of growth of pathogenic *Aer. hydrophila* by the *B. cereus* isolate, in particular competitive exclusion by growth rate, competition for essential nutrients such as glucose and iron, and siderophore production, increase its attractiveness as a probiotic and biological agent for aquaculture. The siderophore producing capability of the *B. cereus* isolate addresses the severe shortage of probiotics able to facilitate competitive exclusion based on iron competition (Patel et al. 2009). The absence of antimicrobial activity is beneficial for application of the *B. cereus* isolate as a biological agent, since the presence of antimicrobial substances in aquaculture systems is undesirable due to increased virulence in disease causing pathogens, negative acceptance by consumers and carryover to the environment (Barker 2000; Jana and Jana 2003). Lack of information on modes of action of biological agents limits the adoption of biological solutions to address the challenges of aquaculture, ultimately perpetuating the use of chemotherapeutic agents (Moriarty, 1997; Moriarty, 1998; Balcázar et al. 2006). The modes of action described here, combined with the *in vitro* and *in vivo* functionality, the ability to reduce the concentration of waste ions in reticulated aquaculture, physiological tolerance to environmental conditions and bio-safety (Lalloo et al. 2007; Lalloo et al. 2008) renders the *B.*

cereus isolate NRRL 100132 as an ideal biological agent to address the many challenges facing modern day intensive aquaculture.

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REFERENCES

Balcázar, J.L., de Blas, I., Ruiz-Zarzuela, I., Cunningham, D., Vendrell, D. and Múzquiz, J.L. (2006) The role of probiotics in aquaculture. *Vet Microbiol* **114**, 173-186.

Barker, G. (2000) Novel methods to reduce disease in aquaculture. *Fish Vet* J **5**:66-71.

Braun, V. and Killmann, H. (1999) Bacterial solutions to the iron-supply problem. *Trends Biochem Sci* **24**, 104-109.

Brunt, J. and Austin, B. (2005) Use of a probiotic to control Latococcosis and streptococcosis in rainbow trout, *Oncorhynchus mykiss* (Walbaum). *J Fish Dis* **28**, 693-701.

Chart, H. and Trust, T.J. (1983) Acquisition of iron by *Aeromonas salmonicida*. *J Bacteriol* **156**, 758-764.

Fast, AW. and Menasveta, P. (2000) Some recent issues and innovations in marine shrimp pond culture. *Reviews Fish Sci* **8**, 151-233.

Fredrickson, A.G. and Stephanopoulos, G. (1981) Microbial competition. *Science* **213**, 972-979.

Gomez-Gill, B., Roque, A. and Turnbull, JF. (2000) The use and selection of probiotic bacteria for use in the culture of larval aquatic organisms. *Aquaculture* **191**, 259-270.

Gram L., Melchiorsen, J., Spanggaard, B., Huber, I. and Nielsen, T. (1999) Inhibition of *Vibrio anguillarum* by *Pseudomonas fluorescens* strain AH2-a possible probiotic treatment of fish. *Appl Environ Microbiol* **65**, 969-973.

Gross, S., Nemirovsky, A., Zilberg, D., Khaimov, A., Brenner, A., Snir, E., Ronen, Z. and Nejidat, A. (2003) Soil nitrifying enrichments as biofilter starters in intensive re-circulating saline water aquaculture. *Aquaculture* **223**, 51-62.

Holzapfel, W.H., Haberer, P., Geisen, R., Björkroth, J. and Schillinger, U. (2001) Taxonomy and important features of probiotic microorganisms in food and nutrition. *Am J Clin Nutr* **73**(suppl), 365S-373S.

Hong, H.A., Duc, L.H. and Cutting, S.M. (2005) The use of bacterial spore formers as probiotics. *FEMS Microbiol Rev* **29**, 813-835.

Irianto, A. and Austin B. (2002) Probiotics in aquaculture. *J Fish Dis* **25**, 633-642.

Jana, B.B. and Jana, S. (2003) The potential and sustainability of aquaculture in India. *J Appl Aquac* **13**, 283-316.

Jeney, Z. and Jeney G. (1995) Recent achievements in studies of common carp (*Cyprinus carpio* L.). *Aquaculture* **129**, 397-420.

Kumar, R., Mukherjee, S.C., Prasad, K.P., Pal, A.K. (2006) Evaluation of *Bacillus subtilis* as a probiotic to Indian major carp *Labeo rohita* (Ham.). *Aquaculture Res* **37**, 1215-1221.

Lalloo, R., Ramchuran, S., Ramduth D., Görgens, J. and Gardiner, N. (2007) Isolation and selection of *Bacillus* spp. as a potential biological agents for enhancement of water quality in culture of ornamental fish. *J Appl Microbiol* **103**, 1471-1479.

Lalloo, R., Maharajh, D., Görgens, J. and Gardiner, N. (2008) Functionality of a *Bacillus cereus* biological agent in response to physiological variables encountered in aquaculture. *Appl Microbiol Biotechnol* **79**, 111-118.

Lalloo, R., Maharajh, D., Görgens, J. and Gardiner, N. (2009) High-density spore production of a *B. cereus* aquaculture biological agent by nutrient supplementation. *Appl Microbiol Biotechnol* **83,** 59–66.

Meza, R.A., Monroy, A.F., Mercado, M., Poutou, R.A., Rodriguez, P. and Pedroza, A.P. (2004) Study of the stability in real time of cryopreserved strain banks. *Universitas Scientiarum* **9**, 35-42.

Milagres, A.M.F., Machuca, A. and Napoleão, D. (1999) Detection of siderophore production from several fungi and bacteria by a modification of chrome azurol S (CAS) agar plate assay. *J Microbiol Meth* **37**, 1-6.

Monteiro, S.M., Clemente, J.J., Henriques, A.O., Gomes, R.J., Carrondo, M.J., Cunha, A.E. (2005) A Procedure for High-Yield Spore Production by *Bacillus subtilis*. *Biotechnol Progr* **21**,1026-1031.

Moriarty, D.W.J. (1998) Control of luminous *Vibrio* species in penaeid aquaculture ponds. *Aquaculture* **164**, 351-358.

Moriarty, D.W.J. (1999) Disease control in shrimp aquaculture with probiotic bacteria. Microbial interactions in aquaculture. N: Bell C.R and Brylinsky, M. (eds) Proceedings of the 8th International Symposium on Microbial Ecology, Canada.

Newaj-Fyzul, A., Adesiyun, A.A., Mutani, A., Ramsubhag, A., Brunt, J., Austin, B. (2007) *Bacillus subtilis* AB1 controls *Aeromonas* infection in rainbow trout (*Oncorhynchus mykiss*, Walbaum). *J Appl Microbiol* **103,** 1699-706.

Park, R.Y., Choi, M.H., Sun, H.Y. and Shin, S.H. (2005) Production of catechol-siderophore and utilization of transferrin-bound iron in *Bacillus cereus*. *Biol Pharm Bull* **28**, 1132-1135.

Patel, A.K., Deshattiwar, M.K., Chaudari, B.L. and Chincholkar, S.B. (2009) Production, purification and chemical characterization of the catecholate siderophore from potent probiotic strains of *Bacillus* app. *Bioresource Technol* **100**, 368-373.

Patterson, J.A. and Burkholder, K.M. (2003) Application of probiotics and probiotics in poultry production. *Poultry Sci* **82**, 627-631.

Pinchuk, I.V., Bressollier, P., Verneuil, B., Fenet, B., Sorokulova, I.B., Megraud, F. and Urdaci, M.C. (2001) *In-vitro* anti-*Helicobacter pylori* activity of the probiotic strain *Bacillus subtilis* 3 is due to the secretion of antibiotics. *Antimicrob. Agents Chemother* **45**, 3156-3161.

Rico-Mora, R., Voltolina, D. and Villaescusa-Celaya, J.A. (1998). Biological control of *Vibrio alginolyticus* in *Skeletonema castatum* (Bacillariophyceae) cultures. *Aquacult Eng* **19**, 1-6.

Schwyn, B. and Neilands, J.B. (1987) Universal chemical assay for the detection and determination of siderophores. *Anal Biochem* **160**, 47-56.

Smith, P. and Davey, S. (1993) Evidence for the competitive exclusion of *Aeromonas salmonicida* from fish with stress-inducible furunculosis by a fluorescent pseudomonad. *J Fish Dis* **16**, 521-524.

Verschuere, L., Rombaut, G., Sorgeloos, P. and Verstraete, W. (2000) Probiotic bacteria as biological control agents in aquaculture. *Microbiol Mol Biol R* **64**, 655-671.

Vine, N.G., Leukes, W.D., Horst, K. (2006) Probiotics in marine larviculture. *FEMS Microbiol Rev* **30**, 404-427.

Wilson, M.K., Abergel, R.J., Raymond, K.N., Arceneaux, J.E.L. and Byers, B.R. (2006) Siderophores of *Bacillus antracis, Bacillus cereus* and *Bacillus thuringiensis*. *Biochem Bioph Res Co* **348**, 320-325.

Winkelmann, G. (2002) Microbial siderophore-mediated transport. *Biochem Soc Trans* **30**, 691-696.

Wolken, W.A.M., Tramper, J. and van der Werf, M.J. (2003) What can spores do for us? *Trends Biotechnol* **21**, 338-345.

TABLE LEGENDS

Table 1 Assessment of different cell preparations for the presence of growth attenuation of *Aer. hydrophila* by *B. cereus*.

Table 2 Growth rate assessment of *B. cereus* and *Aer. hydrophila* cultivated under nutrient limitation.

FIGURE LEGENDS

Figure 1 Decrease in pathogen cell concentration during co-cultivation of *B. cereus* (■) with *Aer. hydrophila* (▲)

Figure 2 Iron (**a)** and glucose (**b)** uptake rates by *B. cereus* (■) and *Aer. hydrophila* (▲)

Figure 3 Growth data based on cell concentration (**a)**; sporulation ratio (**b)** and relative siderophore production **(c)** by *B. cereus* cultivated in synthetic pond water.

Table 1 Assessment of different cell preparations for the growth attenuation of *Aer. hydrophila* by *B. cereus*.

	Growing culture	**Intracellular fraction**	**Extracellular supernatant**
Mid exponential phase	+	-	-
Early stationary phase	+	-	-
Sporulation phase	+	-	-

- No inhibition observed
+ Presence of inhibition

Table 2 Growth rate assessment of *B. cereus* and *Aer. hydrophila* cultivated under nutrient limitation.

Treatment	***B. cereus*** **μ_{max}**	**Std. dev**	***Aer. hydrophila*** **μ_{max}**	**Std. dev**	**Difference in growth rate**	***p* value**
Synthetic pond water	0.041	0.001	0.032	0.000	0.009	0.003
De-ionised water	0.000	0.000	0.000	0.002	0.000	0.374
Low Glucose	0.033	0.001	0.031	0.001	0.002	0.045
Low Nitrite	0.035	0.004	0.031	0.006	0.004	0.473
Low Nitrate	0.032	0.001	0.036	0.004	-0.003	0.210
Low Ammonia	0.033	0.002	0.036	0.002	-0.003	0.266
Low Iron	0.044	0.001	0.033	0.002	0.011	0.001
Low Phosphate	0.000	0.000	0.000	0.000	0.000	$^{n}/_{a}$

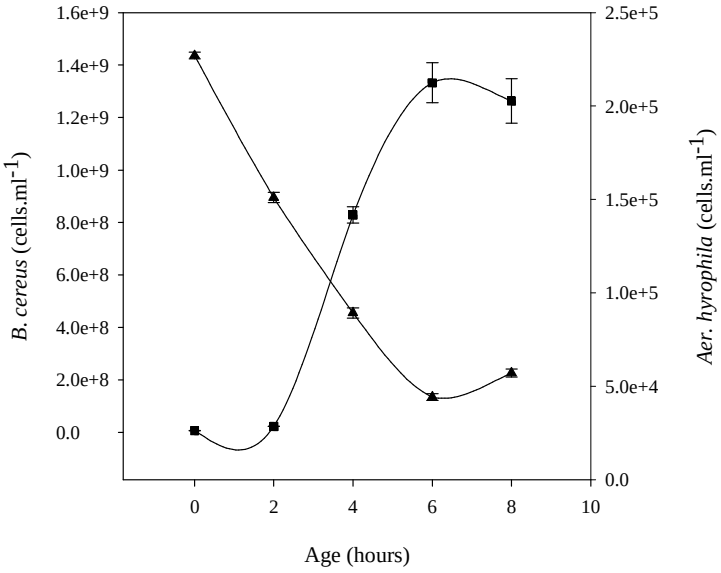

Figure 1 Decrease in pathogen cell concentration during co-cultivation of *B. cereus* (■) with *Aer. hydrophila* (▲)

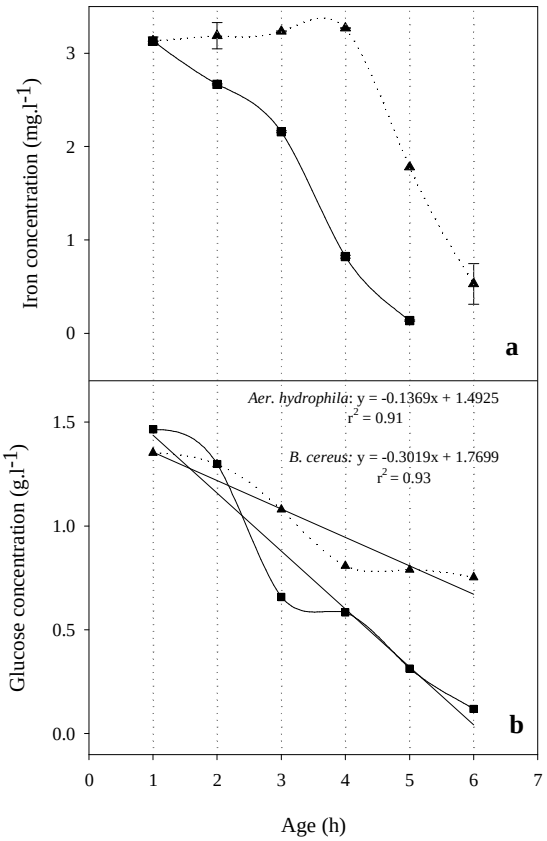

Figure 2 Iron (**a)** and glucose (**b)** uptake rates by *B. cereus* (■) and *Aer. hydrophila* (▲)

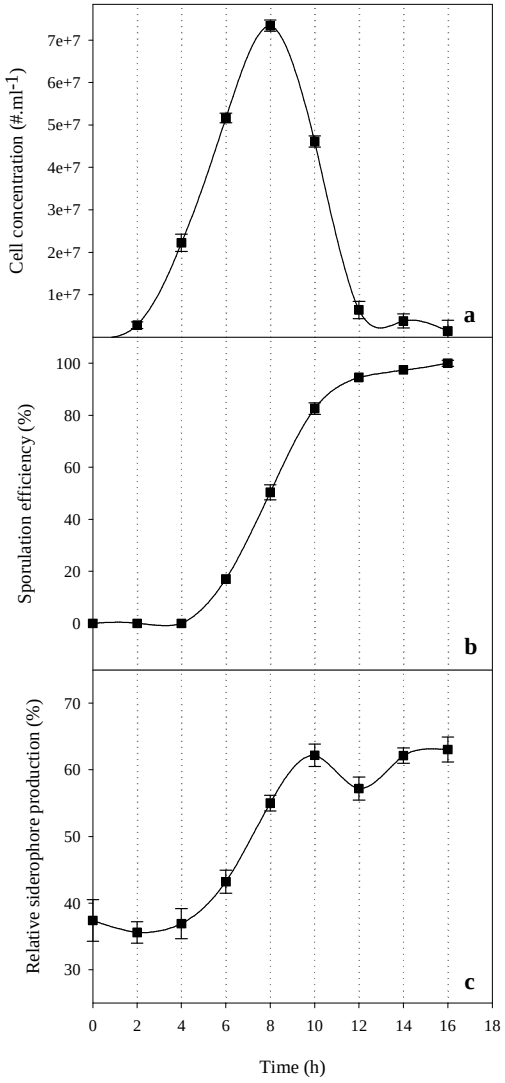
Cell concentration (#.ml-1)
7e+7
6e+7
5e+7
4e+7
3e+7
2e+7
1e+7
a
Sporulation efficiency (%)
100
80
60
40
20
0
b
Relative siderophore production (%)
70
60
50
40
30
c
0
2
4
6
8
10
12
14
16
18
Time (h)

Figure 3 Growth data based on cell concentration (**a)**; sporulation ratio (**b)** and relative siderophore production **(c)** by *B. cereus* cultivated in synthetic pond water.