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ScienceDirect
Aquaculture 261 (2006) 804–808
Aquaculture
www.elsevier.com/locate/aqua-online

Short-chain fatty acids protect gnotobiotic *Artemia franciscana* from pathogenic *Vibrio campbellii*

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Received 28 April 2006; received in revised form 23 June 2006; accepted 23 June 2006

Abstract

Infections caused by antibiotic resistant luminescent vibrios can cause considerable losses in aquaculture. In this study, different short-chain fatty acids were investigated as possible alternative biocontrol agents. The addition of 100 mM formic, acetic, propionic, butyric or valeric acid to the growth medium of a pathogenic *Vibrio campbellii* strain completely inhibited its growth at pH 6. At 10 mM, the growth of the pathogen was delayed, whereas at 1 mM, no effect could be observed. The growth-inhibitory effect was clearly pH-dependent and decreased with increasing pH. An *in vivo* challenge test with gnotobiotic *Artemia franciscana* nauplii revealed that all five short-chain fatty acids protected the shrimp from the pathogenic *V. campbellii* strain. The addition of 20 mM of the short-chain fatty acids to the culture water resulted in a significantly increased survival of infected nauplii, with no difference between the different fatty acids. In conclusion, our data indicate that short-chain fatty acids might be useful as alternative biocontrol agents to treat luminescent vibriosis.

Keywords: Brine shrimp; Short-chain fatty acid; Luminescent vibriosis

1. Introduction

FAO reports consider disease outbreaks as a significant constraint to the development of the aquaculture sector worldwide (Subasinghe et al., 2001). Infections caused by luminescent vibrios are considered to be an important problem in the intensive rearing of molluscs, finfish, lobsters and especially shrimp, where mortality can be as high as 100% (Pass et al., 1987; Alvarez et al., 1998; Lavilla-Pitogo et al., 1998; Diggles et al., 2000). Traditionally, these problems are treated by applying antimicrobials. Many farmers also use antibiotics in a prophylactic way, even when pathogens are not evident (Moriarty, 1999). This practice has resulted in the development of (multiple) antibiotic resistance (Teo et al., 2000; Teo et al., 2002), which makes antibiotic treatments ineffective in controlling the disease (Karunasagar et al., 1994). Therefore, there is an urgent need for alternative control techniques.

Several studies have shown that short-chain fatty acids inhibit the growth of yeast and enterobacteria such as *Salmonella enterica* subsp. *enterica*, *Escherichia coli* and *Shigella flexneri* (Wolin, 1969; Cherrington et al., 1991; Sun et al., 1998; Van Immerseel et al., 2003).

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doi:10.1016/j.aquaculture.2006.06.038

Although the antibacterial mechanism(s) of these compounds are not completely understood, they are capable of exhibiting bacteriostatic and bactericidal properties depending on the physiological status of the organisms and the physicochemical characteristics of the external environment (Ricke, 2003). In the undissociated form, the short-chain fatty acids can pass through the cell membrane of the bacteria and dissociate in the more alkaline cytoplasm, thereby increasing the intracellular concentration of protons (Cherrington et al., 1991). Consequently, the cells have to expend energy in order to maintain the intracellular pH at the optimal level. This energy cannot be used for other metabolic processes and therefore, growth of the cells is inhibited.

In this study, we investigated the bacteriostatic capacities of different short-chain fatty acids *in vitro* and we aimed at testing whether these compounds could be useful as new biocontrol agents for aquaculture, using *Artemia franciscana* as a model.

2. Materials and methods

2.1. Effect of the short-chain fatty acids on growth of V. campbellii LMG21363

Formic, acetic, propionic, butyric and valeric acid were obtained from Sigma-Aldrich (Bornem, Belgium) and dissolved in LB-medium at different concentrations (1, 10

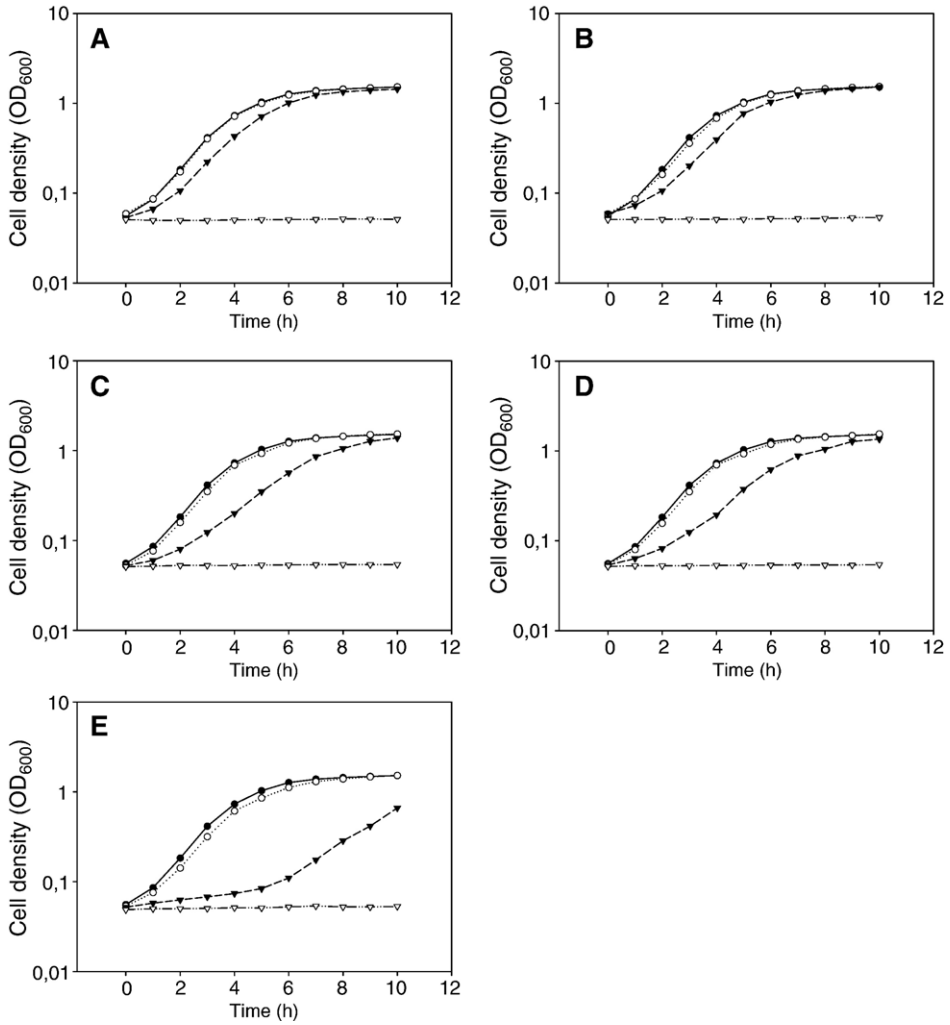

Fig. 1. Growth of *V. campbellii* LMG21363 (OD_{600}) in LB-medium at pH 6 with and without different short-chain fatty acids, added at different concentrations. The fatty acids tested were formic acid (panel A), acetic acid (panel B), propionic acid (panel C), butyric acid (panel D) and valeric acid (panel E). The data points are the mean values of 3 replicates. Symbols are —●—: no fatty acid; ·····○·····: 1 mM, – – –▼– – –: 10 mM, –··–▽·–··: 100 mM.

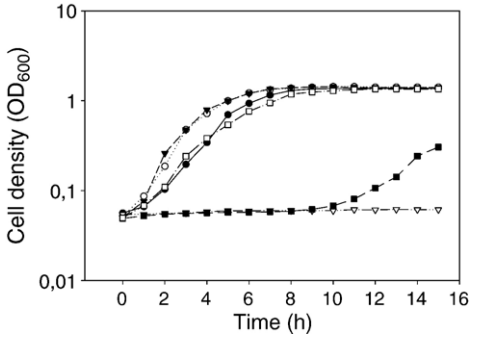

Fig. 2. Growth of *V. campbellii* LMG21363 (OD_{600}) in LB-medium at pH 5, 6 or 7, with and without 20 mM valeric acid. The data points are the mean values of 3 replicates. Symbols are —●—: control, pH 5; ·····○·····: control, pH 6; – – –▼– – –: control, pH7; –··–▽·–··: valerate, pH 5; – –■–: valerate, pH 6; – –□– –: valerate, pH 7.

and 100 mM). The pH of the solutions was adjusted to 6 after which they were filter sterilised. The sterile media were inoculated with a *V. campbellii* culture (diluted to an OD_{600} of 1) at 20 µl culture per ml medium. The suspensions were incubated at 28 °C for 10 h and the optical density (600 nm) was measured every hour. In a second experiment, 20 mM solutions of formic and valeric acid in LB-medium were prepared after which the pH was adjusted to 5, 6 or 7, respectively. The solutions were inoculated with *V. campbellii* and incubated at 28 °C for 15 h. Again, the optical density (600 nm) was measured every hour.

2.2. In vivo challenge tests

In vivo challenge tests were performed as described previously (Defoirdt et al., 2005). Briefly, *Artemia* cysts were decapsulated and hatched for 30 h at 28 °C. After hatching, groups of 20 nauplii were transferred to sterile falcon tubes containing a sterile 20 mM solution of the short-chain fatty acids in filtered and autoclaved artificial seawater (pH was adjusted to 7). The nauplii were fed with an autoclaved suspension of *Aeromonas hydrophila* LVS3 (at approx. 10^7 CFU ml^{-1}) and *V. campbellii* LMG21363 was added to the culture water at approx. 10^5 CFU ml^{-1}. The falcon tubes with the nauplii were incubated on a rotor at 28 °C and *Artemia* survival was measured 2 days after the addition of the pathogen.

2.3. Statistics

Different treatments were compared by independent samples *t*-tests, using the SPSS software, version 12.0. Differences were considered significant if the *P*-value of the *t*-test was below 0.05.

3. Results and discussion

3.1. Effect of short-chain fatty acids on growth of V. campbellii LMG21363

In an initial experiment, we investigated the effect of formic, acetic, propionic, butyric and valeric acid on the growth of the shrimp pathogen *V. campbellii* LMG21363 in liquid growth medium. At pH 6, all fatty acids completely inhibited the growth of *V. campbellii* at a concentration of 100 mM (Fig. 1), whereas there was no effect on growth of the pathogen at 1 mM and at 10 mM, the growth of the strain was not completely inhibited but showed a longer lag phase than without fatty acids. Our data indicate that the concentrations of short-chain fatty acids needed to inhibit the growth of *V. campbellii* are in the same order as those for enteric bacteria. Indeed, Wolin (1969) reported a partial inhibition of the growth of *E. coli* by approximately 10 mM of short-chain fatty acid at pH 6 and Van Immerseel et al. (2003) also reported that the growth of *S. enterica* subsp. *enterica* was completely inhibited by 100 mM fatty acid at pH 6.

In a second *in vitro* experiment, we studied the influence of pH on the growth-inhibitory activity of the fatty acids. For both fatty acids (at a concentration of 20 mM), the growth-inhibitory effect clearly decreased with increasing pH. At pH 5, growth was completely inhibited; at pH 6, growth was delayed and at pH 7, no inhibition could be observed. The results for valeric acid are shown in Fig. 2. Similar pH-dependent effects have been reported for the inhibition of the growth of *E. coli*, and *S. enterica* subsp. *enterica* by short-chain fatty acids (Wolin, 1969; McHan and Shotts, 1993; Van Immerseel et al., 2003). The pH-dependent effect can be

Table 1
Percentage survival of *Artemia* nauplii (mean±standard error of three replicates) after 2 days challenge with *V. campbellii* LMG21363

Treatment	Survival (%)
Control	85±3
LMG21363	20±3
LMG21363+formic acid (20 mM)	45±5*
LMG21363+acetic acid (20 mM)	48±7*
LMG21363+propionic acid (20 mM)	48±6*
LMG21363+butyric acid (20 mM)	42±2**
LMG21363+valeric acid (20 mM)	47±7*

The short-chain fatty acids were added to the *Artemia* culture water at the start of the experiment.
* Significant difference in survival with infected nauplii without the addition of fatty acid ($P<0.05$).
** Significant difference in survival with infected nauplii without the addition of fatty acid ($P<0.01$).

explained by the fact that the fatty acids can pass the cell membrane only in their undissociated form, which is more dominant at lower pH (Sun et al., 1998).

3.2. Effect of the short-chain fatty acids on survival of *Artemia nauplii* infected with *V. campbellii* LMG21363

Since the short-chain fatty acids were found to inhibit the growth of *V. campbellii* LMG21363, we investigated whether these compounds could protect *Artemia* nauplii from the pathogen in an *in vivo* challenge test. The fatty acids significantly enhanced the survival of infected nauplii (Table 1), with no significant difference between the different fatty acids. Further increasing the fatty acid concentration to 100 mM did not further increase the survival of the nauplii (data not shown). We presume that the addition of the short-chain fatty acids in the culture water limited pathogen colonisation by conversion of the acids into their respective antibacterial forms in the digestive tract of *Artemia*. However, apart from inhibiting the growth of *V. campbellii*, the protection offered by the short-chain fatty acids might also be due to a feed effect. Indeed, it has been shown before that the susceptibility of *Artemia* nauplii to pathogenic bacteria decreases if they are fed a better quality feed (Marques et al., 2005). Short-chain fatty acids are known to provide mammals (and especially the mammalian colonic mucosa) with energy (Topping and Clifton, 2001), which might also be the case for *Artemia*. However, we did not observe higher survival in starved nauplii treated with short-chain fatty acids when compared to untreated nauplii (data not shown), which indicates that the amount of energy obtained from the fatty acids is rather limited.

Short-chain fatty acids have previously been shown to inhibit or decrease the growth of *Salmonella* in chickens (Waldroup et al., 1995; van der Wielen et al., 2000; Van Immerseel et al., 2005). In fact, these compounds are already used in commercial mixtures to control *Salmonella* in poultry (Van Immerseel et al., 2002). Interestingly, Vázquez et al. (2005) have recently shown that acetic acid had an inhibitory effect towards *Vibrio alginolyticus*, *Vibrio pelagius* and *Vibrio anguillarum*. Our data indicate that short-chain fatty acids might also be useful for controlling luminescent vibriosis in aquaculture. However, it would economically and practically not be feasible to dose the fatty acids in the culture water of an aquaculture system since the effective concentrations are rather high. Therefore, further research will be necessary in order to provide more elegant methods to deliver the short-chain fatty acids to the digestive tract of the animals. One possibility could be the application of microencapsulated fatty acid particles (as used in poultry diets; Van Immerseel et al., 2005). Unfortunately, we were not able to test the microencapsulated fatty acid particles used in poultry diets in our model system since those particles are too large to be ingested by *Artemia*.

Acknowledgements

This work was supported by the “Instituut voor de aanmoediging van Innovatie door Wetenschap en Technologie in Vlaanderen” (IWT grant no. 33205) and the “Fonds voor Wetenschappelijk Onderzoek” (project no. G0230.02N). Special thanks to Greet Dewaele for critically reading the manuscript.

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