The use of probiotics to protect fish against rainbow trout fry syndrome

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Abstract

Flavobacterium psychrophilum causes high levels of mortalities in aquaculture, especially in salmonid culture during the early life stages of rainbow trout (*Oncorhynchus mykiss*, Walbaum) [e.g. 1]. Although antibiotics have been successful in treating *F. psychrophilum* infections there can be problems with resistant strains. Probiotics, defined as beneficial live micro-organisms when administered to a host at an effective dose, provide a potential alternative strategy for reducing disease outbreaks in aquaculture systems. The specific action of a probiotic is often based on its antagonistic activity at the site of colonisation on the host's surface, enhancement of the host's immune response, competition for nutrients, and/or production of antimicrobial substances towards the pathogen [2]. In particular, the inhibitory effect of probiotic bacteria on fish pathogens has been demonstrated under iron depleted conditions, resulting in the production of siderophores by the probiotic in the absence of iron [3,4]. Siderophores are high affinity iron acquisition molecules produced by bacteria, giving them a competitive advantage in iron scarce environments [5]. One of the probable modes of action of *Pseudomonas* spp. (as used here) is thought to be through their ability to produce siderophores [6].

One of the reasons for the high mortality seen in fry during RTFS outbreaks could be the lack of a developed adaptive immune response in these young fish. However, the young fish may be protected by innate immunity [7], and thus the effect of immunostimulants and probiotics may be beneficial in improving their innate disease resistance. Moreover, natural antibodies are present in the serum of mammals, birds and fish without any apparent antigenic stimulation, and are thought to be an important innate humoral defence against invading pathogens [8,9]. A number of immunological parameters have been found to be stimulated in fish fed with probiotics [for review see 10], including total serum IgM levels [11–13].

The aim of the present study was to evaluate the antagonist activity of *Pseudomonas* M174 and M162 against *F. psychrophilum*, and to examine their possible modes of action. The materials and methods used in the analysis of samples are described in detail in [14,15].

The potential of using probiotic bacteria *Pseudomonas* M174 and M162, isolated from the surface of rainbow trout eggs, to reduce *F. psychrophilum* infections in rainbow trout fry was investigated. Both bacteria were shown to inhibit the growth of *F. psychrophilum in vitro*. When these were tested *in vivo*, they were shown to be harmless to fish, and were both

antagonistic against *F. psychrophilum* with relative percentage survival (RPS) of 39.3% and 49.1%, respectively, when *Pseudomonas* M162 and M174 were applied as probiotics in feed. Although *Pseudomonas* M162 and M174 were isolated simultaneously from the same batch of rainbow trout eggs they were shown to have genotypic (97% similarity by 16S rRNA gene sequence) and phenotypic differences (e.g. siderophore production). The results suggest that both M162 and M174 produce siderophores, but with different absorbance spectra for their corresponding filtered culture supernatants [14]. Furthermore, siderophores of M174 were shown to inhibit the growth of *F. psychrophilum*, while no inhibition was observed with supernatants from iron-depleted cultures of M162. It is known that competition in iron acquisition exists between the probiotic and the pathogen, and *F. psychrophilum* is known to produce siderophores [16]. However, this iron acquisition can differ between serotypes of *F. psychrophilum* and is relatively weak compared to other bacterial pathogens affecting fish [6]. Although siderophore production by a probiotic and its depletion of iron resources seems to be an effective method of inhibiting *F. psychrophilum* growth [6, 14], it does not appear to be the mode of the action of M162 against *F. psychrophilum.*

Both *Pseudomonas* tested here were shown to colonise the gut, with the number of probiotic bacteria decreasing after probiotic supplementation ceased. The results of the present study are in agreement with observations made by Balcázar et al. [17] and Nikoskelainen et al. [11] for lactic acid bacteria (LAB) indicating that permanent colonisation by externally applied probiotic strains is rarely achieved. Several studies have suggested that the attachment and colonization of probiotics on the intestine may lead to stimulation of the innate immune response of the fish [12, 18]. In one study feeding *Bacillus subtilis* to rainbow trout, a higher numbers of leukocytes were found at week 3 in probiotic-fed fish compared to the control group [19]. Significantly higher levels of leukocytes were also found in the present study at week 3 sampling with probiotic *Pseudomonas* 162 (Table 1).

Both bacteria had an immunostimulatory effect on their host, especially through the innate immune response of the fish. The respiratory burst activity of head kidney macrophages was enhanced in fish fed with *Pseudomonas* M174, while *Pseudomonas* M162 did not exhibit any significant change in macrophage respiratory burst activity compared to the control group. The latter group did, however, appear to have a greater immunostimulatory effect on the fish, stimulating their peripheral blood leukocyte counts, serum lysozyme activity and total serum immunoglobulin levels after three weeks of feeding the probiotic (Table 1) [15]. The immunostimulatory effect of M162 was confirmed at a gene expression level through microarray analysis.

Díaz-Rosales et al. [20] reported that two probiotic bacteria originating within the same genus had different effects on the respiratory burst activity of Senegalese sole (*Solea senegalensis*), but both probiotics improved resistance against *Photobacterium damselae* subsp. *piscicida*. Although the innate immunology parameters induced by M162 and M174 were not totally comparable as immunological parameters were only analysed after 2 weeks of feeding with M174, these studies do suggest that although M162 and M174 are from the same origin, and both improve resistance to RTFS, their mode of action seems to differ and further studies combining these probiotics could be useful.

Table 1. Haematological and innate immunological analyses (Average ± SD) of blood samples taken on weeks 2 and 3 following treatment with *Pseudomonas* M162.

Treatment	Erythrocytes (1×10^8 ml^{-1})	Leukocytes (1×10^7 ml^{-1})	Respiratory activity	Phagocytic activity (%)	Lysozyme activity (U^{-1} min^{-1} ml^{-1})
Week 2					
Probiotic	10.6±2.4 (n=12)	4.7±0.97* (n=12)	0.7±0.49 (n=5)	22.4±8.9 (n=10)	119.5±61.2 (n=12)
Control	9.2±1.99 (n=10)	5.7±0.97 (n=10)	0.4±0.3 (n=10)	20.8±10.9 (n=11)	172.2±171.7 (n=9)
Week 3					
Probiotic	9.5±1.3 (n=12)	7.6±2.6* (n=12)	0.03±0.001 (n=12)	12.6±3.7 (n=11)	893.3±622.7* (n=12)
Control	10.3±1.2 (n=12)	5.9±0.9 (n=12)	0.03±0.006 (n=12)	17.7±9.4 (n=11)	395.8±332.9 (n=12)

*Statistically significantly different from control ($P \leq 0.05$, *t*-test)

When selecting potential probiotics both safety and efficacy should be considered, and screening their effectiveness *in vitro* and their applicability *in vivo* is essential [2]. In the present study both *Pseudomonas* M162 and M174 were demonstrated to be potential probiotics; they had no unfavourable effects on the health of the fish, they survived in the gastrointestinal track of the fish and inhibited the growth of *F. psychrophilum in vitro,* and improved resistance to it *in vivo*. The modes of action of the two probiotics, however, appeared to differ in that although both produced siderophores, only the M174 siderophores were shown to inhibit the growth of *F. psychrophilum*, while no inhibition was observed with supernatants from iron-depleted cultures of M162. In addition, feeding fish with *Pseudomonas* M162-supplemented feed resulted in a greater immunostimulatory effect on the fish than M174, stimulating peripheral blood leukocyte counts, serum lysozyme activity and total serum immunoglobulin levels.

Rainbow trout fry syndrome causes high mortalities during the early life stages of the fish, partly because their adaptive immunity has not yet fully developed. Thus, immunomodulation by probiotics could be an effective prophylactic method against RTFS.

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