Aquaculture Research

Aquaculture Research, 2011, **42**, 693–703 doi: 10.1111/j.1365-2109.2010.02744.x

Effects of probiotic bacteria on growth parameters and immune defence in Eurasian perch *Perca fluviatilis* L. larvae under intensive culture conditions

SNM Mandiki, Sylvain Milla, Neil Wang, Gersande Blanchard, Thierry Djonkack, Stéphane Tanascaux & Patrick Kestemont

Research Unit in Organismal Biology (URBO), University of Namur, Namur, Belgium

Correspondence: S N M Mandiki, Research Unit in Organismal Biology (URBO), University of Namur, Rue de Bruxelles 61, B-5000 Namur, Belgium. E-mail: robert.mandiki@fundp.ac.be

Abstract

Two experiments were conducted to examine whether a mixture of bacteria composed of *Bacillus licheniformis*, *Bacillus subtilis* and *Bacillus pumilus* has a beneficial impact on survival, growth, lysozyme activity and total immunoglobulins (Ig) in perch larvae. In experiment 1, control fish were compared with fish receiving bacteria (4.93×10^7 CFU L^{-1} day^{-1}) through tank water or both through tank water and *Artemia* metanauplii enriched with two bacteria doses (2×10^{10} or 4×10^{10} CFU L^{-1}). Treatments were performed in 40 L triplicate tanks of 200 fish [initial body weight (IBW) = 2.3 mg] each, placed in separated recirculating water systems. Experiment 2 evaluated the combining effects of bacteria feeding and densities (10 vs. 20 fish L^{-1}, IBW = 9.5 mg). In both experiments, the bacteria added to live food or both live food and water induced higher growth rate at the highest dietary dose and this was associated with higher Ig levels on days 14 and 28. In experiment 2, higher survival was associated to lower type-2 cannibalism (ingestion of whole prey) at the two dietary bacteria doses, and such positive effect was observed for fish fed bacteria at high density. Bacteria administration through water had no effect on growth and survival. In conclusion, the results show that high dietary concentrations of the mixture of bacteria used have potential stimulating impact on growth, survival, lysozyme activity and total Ig. But, further investigations are needed to demonstrate the optimal doses for emphasizing immune defence status.

Keywords: *Bacillus* probiotics, Eurasian perch larvae, growth performance, immune defence

Introduction

Eurasian perch has been identified as a valuable candidate for aquaculture diversification in Europe but the profitability of its farming is still compromised by a high mortality rate during the larval and early juvenile developmental stages. Results from several studies and pilot-scale farming have demonstrated efficient reproductive performance in ponds, mesocosms and recirculating tank systems after natural spawning or ovulation and spawning induced by photothermal manipulations (Migaud, Fontaine, Sulistyo, Kestemont & Gardeur 2002; Migaud, Mandiki, Gardeur, Kestemont, Bromage & Fontaine 2003; Fontaine, Pereira, Wang & Marie 2006; Wang, Gardeur, Henrotte, Marie, Kestemont & Fontaine 2006; Policar, Kouril, Stejskal & Hamackova 2008). But despite a high number of larvae obtained at hatching, survival is low after 40 days of larviculture, ranging from 5% to 20%, depending on the method of fingerling production (Kestemont & Mélard 2000; Kestemont, Rougeot, Musil & Toner 2008). The occurrence of a high cannibalism rate emerging around day 10 post hatching (p-h) has been reported as one of the most important factors affecting survival in Eurasian perch (Baras, Kestemont & Mélard 2003; Babiak, Mandiki, Ratsinjomanana & Kestemont 2004; Mandiki, Babiak, Krol, Rasolo & Kestemont 2007; Kestemont *et al.* 2008). It has also been reported that high stocking densities mitigate cannibalism rates in larvae, thus resulting in a higher survival without compromising growth (Baras *et al.* 2003; Kestemont, Jourdan, Houbart, Mélard, Paspatis, Fontaine, Cuvier, Kentouri & Baras 2003). As for other fish species, bacterial, parasitic and fungal diseases have been also reported as another important factor inducing high

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mortalities during the early developmental stages of Eurasian perch (Grignard, Mélard & Kestemont 1996; Wahli, Burr, Pugovkin, Mueller & Frey 2005; Kempter, Piasecki, Wieski & Krawczyk 2006; Rodger, Toner & Vandecan 2008).

Compared with old aquaculture fish families, such as cyprinids and salmonids, therapeutic or prophylactic strategies for controlling most of outbreaks in the Eurasian perch culture are not yet established. Nevertheless, some antibiotics such as orally administered oxytetracycline have been used with success against epitheliocystis or furunculosis, caused by chlamydia-like organisms or *Aeromonas* spp. (Wahli *et al.* 2005; Rodger *et al.* 2008). Preliminary studies also indicated that perch fry appears more vulnerable to some classic prophylactic strategies used for salmonids, such as repeated formalin baths (Grignard *et al.* 1996; Kempter *et al.* 2006; Rodger *et al.* 2008). Therefore, the development of alternative prophylactic strategies is of high interest for the development of Eurasian perch culture. Various probiotics have been tested in animal production not only for effective prophylactic purposes but also to mitigate the emergence of antibiotic resistance, as well as the spreading of antibiotics in the aquatic environment (Musa, Wu, Zhu, Seri & Zhu 2009). In previous studies conducted to identify probiotics for fish culture, various non-antagonistic bacteria belonging to the *Bacillus* and *Lactobacillus* genus appear attractive in providing efficient prophylactic strategies but mainly salmonid fish were tested (see review by Kesarcodi-watson, Kaspar, Lategan & Gibson 2008; Ringo, Lovmo, Kristiansen, Bakken, Salinas, Myklebust, Olsen & Mayhew 2010). Thereafter, the identified probiotic bacteria were compared with other bacterial species and tested in other fish species. Beneficial impact of *Bacillus subtilis* on survival and growth parameters was demonstrated in Indian major carp *Labeo rohita* H. (Kumar, Mukherjee, Prasad & Pal 2006). The same result was obtained in Nile tilapia *Oreochromis niloticus* L. using *Bacillus pumilus* and *B. subtilis* (El-haroun, Goda & Chowdhury 2006; Aly, Mohamed & John 2008). *Lactobacillus acidophilus* has been also shown to promote growth rate in African catfish (Al-dohail, Hashim & Aliyu-paiko 2009), and the same positive impact has been reported with other bacteria such as, *Vibrio* spp. in fingerlings of sea bass *Dicentrarcus labrax* L. and Senegalese sole *Solea senegalensis* K. (Makridis, Martins, Reis & Dini 2008; Rengpipat, Rueangruklikhit & Piyatiratitivorakul 2008). Other types of probiotics based on either pure yeast preparations or mixtures of bacteria and yeast (Aly *et al.* 2008; Abdelhamid, Mehrin, El-barbary, Ibrahim & Abdel-wahab 2009; He, Zhou, Liu, Shi, Yao, Ringo & Yoon 2009) were also shown to enhance survival and growth rates in tilapia and African catfish. Most of the probiotics tested also markedly induced changes in haematological parameters or some immune compounds and in some cases, improved disease resistance (Aly *et al.* 2008; Kesarcodi-Watson *et al.* 2008) but the mechanisms by which probiotic products influence survival, growth or immunity are not well elucidated in fish. During the early developmental stages, probiotic products are administered by enrichment of live food, added to the dry diet or to the culture water (Panigrahi, Kiron, Puangkaew, Kobayashi, Satoh & Sugita 2005; Kesarcodi-Watson *et al.* 2008; Apun-molina, Santamaria-miranda, Luna-gonzalez, Martinez-diaz & Rojas-contreras 2009). Inappropriate doses or choice of bacterial species may result in detrimental effects on the balance of friendly and pathogenic microflora in the gastrointestinal tract, and thus on the health status depending on the fish species (Lategan, Torpy & Gibson 2004; Rastall 2004; Wang, Li & Lin 2008). To our knowledge, the administration strategy and actions of probiotics have not yet been documented in percid fish. Therefore, the present study was conducted to examine whether a mixture of different *Bacillus* strains (namely *Bacillus licheniformis*, *B. subtilis* and *B. pumilus*) has a beneficial impact on animal husbandry and immune variables in Eurasian perch larvae with regard to different rearing density levels.

Materials and methods

Fish and rearing conditions

Two experiments were carried out with postlarvae originating from wild breeders captured from the Meuse River near Namur, Belgium (50°25′N; 5°2′E), in early April 2008 and 2009 and kept in tanks at 14 °C. For each experiment, a group of three females and 10 males were followed daily for spawning. Fertilized eggs were collected and incubated in a flow-through water recirculating system at 16 °C. After hatching, the larvae were reared at 19–20 °C in water with dissolved oxygen at 7.3–8.4 mg O_2 L^{-1}, NO_2^- and NH_3 at 0.02–0.05 and 0.04–0.08 mg L^{-1} respectively. To standardize the feeding behaviour, larvae were fed *Artemia nauplii* AF strain (INVE Aquaculture, Dendermonde, Belgium) nine times during the light hours from the second day p-h to day 14 or 18 p-h, before the bacteria administration.

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Experiment 1

The probiotic product was a commercial mixture (SANOLIFE MIC-F®, INVE Aquaculture) composed of different *Bacillus* (B) species, namely *B. licheniformis*, *B. subtilis* and *B. pumilus*. Two administration modes of bacteria were compared starting on day 14 p-h, which was considered as day 0 (D0). Fish [initial body weight (IBW) = 2.3 ± 0.8 mg] were submitted to four experimental treatments. Control fish (B0) were fed normal-size *Artemia* metanauplii without probiotics either in live food or in tank water. They were compared with fish that received the probiotic bacteria through tank water (BW), and that were fed normal-size *Artemia* metanauplii without enrichment by probiotics. Other fish received probiotic bacteria both through the tank water and were fed *Artemia* metanauplii enriched with either 2×10^{10} (BWF2) or 4×10^{10} (BWF4) CFU L^{-1} of incubation medium. The experimental protocol is summarized in Table 1.

The treatments were carried out in 40 L triplicate tanks of 200 fish each, placed in separated recirculating water systems. Fish were fed in excess every hour between 09:00 and 19:00 hours for a 28-day period. Perch larvae can be fed live food since hatching to the post-larval stage without compromising effects on growth, and high mortality rate has been reported when they are weaned between the third or fourth week after hatching due to difficulties of adaptation to dry food (Kestemont *et al.* 2008). To avoid an eventual overlapping of this problem in the evaluation of the effect of probiotic bacteria on growth and survival, live food composed of *Artemia* metanauplii was used over the 28-day period for the two experiments.

Experiment 2

The bacteria administration starts on day 18 p-h (D0). Seven experimental treatments were set. On the one hand, a set of three conditions was tested for which postlarvae (IBW = 9.5 ± 4 mg) were reared at 10 fish L^{-1}. First, control groups did not receive any probiotics either through live food or through water (B010). In the two other groups, fish received the probiotic bacteria through food (BF) composed of normal-size *Artemia* metanauplii enriched with two bacterial doses: either 2×10^{10} (BF2/10) or 4×10^{10} (BF4/10) CFU L^{-1} of the incubation medium. On the other hand, a set of four treatments were tested. Fish were reared at two stocking densities (10 vs. 20 larvae L^{-1}), and received the probiotic bacteria through water (BW). As in experiment 1, 4.93×10^{7} CFU L^{-1} day^{-1} were mixed with the water for the two densities (BW10 vs. BW20). These treatments were compared with fish reared at the same densities, and which were fed *Artemia* metanauplii enriched by probiotic bacteria (2×10^{10} CFU L^{-1} incubation medium) in addition to water (BF2W10 vs. BF2W20). The experimental protocol is summarized in Table 2.

The treatments were carried out in 40 L triplicate tanks placed in separated recirculating water systems for the two doses, and fish were fed in excess every hour between 09:00 and 19:00 hours for a 28-day period as in experiment 1.

For the two experiments, *Artemia* cysts were incubated over 48 h (30 g L^{-1}, 2000 lx, and 28 °C) to produce instar II nauplii (metanauplii). Then, equal amounts of these metanauplii were incubated without or with one of the two doses of *Bacillus* mixture during 1 h. The metanauplii were thereafter rinsed and provided to fish as live food. The total number of germs in the tank water was determined using the colony countering method according to the reference standard ISO 6222. The concentration of total germs counted as daily addition to a recirculating rearing

Table 1 Summary of the experimental protocol in experiment 1

Treatments	Administration mode and bacteria doses
B0	Control without probiotics
BW	Water: 4.93×10^{7} CFU L^{-1} day^{-1}
BWF2	Water: 4.93×10^{7} CFU L^{-1} day^{-1}+live food: 2×10^{10} CFU L^{-1}
BWF4	Water: 4.93×10^{7} CFU L^{-1} day^{-1}+live food: 4×10^{10} CFU L^{-1}

Table 2 Summary of the experimental protocol in experiment 2

Treatments	Stocking density	Administration mode and bacteria doses
B010	10 fish L^{-1}	Control without probiotics
BF2/10	10 fish L^{-1}	Live food: 2×10^{10} CFU L^{-1}
BF4/10	10 fish L^{-1}	Live food: 4×10^{10} CFU L^{-1}
BW10	10 fish L^{-1}	Water: 4.93×10^{7} CFU L^{-1} day^{-1}
BW20	20 fish L^{-1}	Water: 4.93×10^{7} CFU L^{-1} day^{-1}
BF2W10	10 fish L^{-1}	Live food: 2×10^{10} CFU L^{-1}+water: 4.93×10^{7} CFU L^{-1} day^{-1}
BF2W20	20 fish L^{-1}	Live food: 2×10^{10} CFU L^{-1}+water: 4.93×10^{7} CFU L^{-1} day^{-1}

system (Tables 1 and 2) was considered as the difference between counting in the out flow water and at the tap water in flow of that rearing system.

Measurements of survival, growth and immune variables

For each replicate, dead fish were counted and removed daily, and all the individuals were counted at the end of each experiment. Then, survival (%) was calculated according to $100 \times N_f/N_i$, and mortality caused by type-2 cannibalism (Can II%, ingestion of whole prey) as $100 \times ((N_i - (N_f - N_d))/N_i)$, where N_i and N_f are the initial and final numbers of fish, respectively, and N_d is the number of dead fish observed during the experiment. Type-1 cannibalism (percentage of truncated victims with posterior part damaged or tails bitten off) was not measured because the two experiments were carried out during the post-larval stage, and it has been shown that under intensive culture, type-1 cannibalism emerges in Eurasian perch larvae around 10-day p-h and is switching almost completely to the type-2 cannibalism within the next week (Baras *et al.* 2003). Moreover, type-2 cannibalism has been found more intense than type-1 for postlarvae perch (Babiak *et al.* 2004; Mandiki *et al.* 2007).

Before the bacteria administration (day 0), 50 larvae from the whole population were weighed individually, and 30 fish per replicate were considered for intermediate (day 14) and final (day 28) weight measurements. Specific growth rate (SGR% day^{-1}) was calculated as $100 \times (\ln W_2 - \ln W_1)/\Delta T$, where W_1 and W_2 are the initial (IBW) and final (FBW) body weights, respectively, and ΔT is the time interval (days) between weightings. On day 28, ΔT was the entire period of bacteria administration (D0–D28). Coefficients of variation in body weight (CV, %) were calculated as: $100 \times SD_A/A$, where A is the average body weight and SD_A = standard deviation of average body weight. Then, the final weight heterogeneity (FWH) was calculated as $CV_{FBW}\%/CV_{IBW}\%$.

The 30 larvae per replicate used for intermediate and final weight measurements were stored at − 80 °C for analyses of total proteins, lysozyme activity and total immunoglobulin (Ig) in the larval homogenates. These latter parameters were selected because of their accuracy to account for non-specific and specific immune status, respectively, in fish. The larval samplings were homogenized using phosphate-buffered saline [v/v (phosphate Na 0.067 M and triton X-100 0.1%)/(ethanol 95% and acetic acid 1%)] pH = 6.2, centrifuged at 6000 *g* for 10 min and the supernatants were used for immunological analyses. Total proteins in the larval homogenates were determined by the Bradford method (Bradford 1976; Kilkowski & Gross 1999). The lysozyme activity protocol was adapted from Ellis (1990). In 96 microplates wells, the lysozyme activity assay was initiated by mixing 10 µL of the larval homogenate supernatant with 250 µL of lyophilized *Micrococcus lysodeikticus* (SIGMA, Brussels Belgium) suspension at a concentration of 0.6 mg mL^{-1} in phosphate buffer saline (pH = 6.2). The difference in absorbance at 450 nm monitored between 0 and 15 min was used to calculate lysozyme activity in units with 1 U representing the amount of lysozyme that caused a 0.001 decrease in absorbance per minute. The lysozyme activity was calculated in units per microgram of larval homogenate proteins. Concentration of total Ig in the larval homogenates was measured by the method of Siwicki and Anderson (1993) and modified by Dautremepuits, Stéphane Betoulle, Paris-palacios and Vernet (2004). Immunoglobulins were precipitated with 10 000 kDa polyethylene glycol (PEG, SIGMA). Larval homogenate supernatant was mixed with an equal volume of 12% PEG solution for 2 h at room temperature under constant shaking. After centrifugation at 1000 *g* for 10 min, the supernatant was collected and assayed for its protein concentration. The total Ig concentration (mg g^{-1} tissue) was calculated by subtracting this value from the total protein concentration in the supernatant.

Statistical analyses

Values are mean ± SD, and data expressed in percentages were log-transformed before statistical calculations. In experiment 1, analysis of variance (ANOVA)-I was used to test the main effect of administration mode on the husbandry variables, while ANOVA-II was considered for the effect of administration mode and its duration on immune variables. In experiment 2, ANOVA-II was used to test the effect of administration mode and its interaction with stocking density on the husbandry variables, and a third level (ANOVA-III) was considered as the duration of bacteria administration. Significance of differences between groups was estimated using the Scheffe's multiple range tests with a significance level at $P < 0.05$. Calculations were performed using STATISTICA software (STATSOFT, Tulsa, OK, USA).

Results

Husbandry variables

In experiment 1 (Table 3), bacteria administration through live food and water (BFW) induced significant ($P < 0.05$) growth rate when compared with control fish (B0) and fish that received bacteria through tank water (BW). The stimulating growth response was observed at the highest dietary dose (FB4W) on day 14, and was sustained over the 28-day period of the probiotic treatment. In experiment 2 (Table 4), the stimulating growth response was observed only on day 28 when probiotic bacteria were administered through only live food (BF) at the highest dose (BF4/10). In both experiments, low bacteria dose in live food did not affect growth as for the bacteria administration through tank water (Tables 3–4). Final weight heterogeneity was significantly ($P < 0.05$) reduced at the end of both experiments (Tables 3–4) in fish fed bacteria through combining live food and tank water or through live food only, except for BF2W10 fish in experiment 2. In contrast to the dietary administration mode, water bacteria administration through the tank water did not affect FWH in both experiments. Growth data of experiment 2 did not show a marked effect of stocking density, except for a low FWH ($P < 0.05$) in BF2W20 fish that received bacteria through both live food and in tank water, and reared at the highest density level (Table 4).

In experiment 1, survival was not affected by the probiotic bacteria whatever the administration mode or the dietary dose (Table 3). However, mortality rate caused by type-2 cannibalism was significantly reduced ($P < 0.05$) by the dietary bacteria administration not by the administration through tank water. High survival associated with low type-2 cannibalism ($P < 0.05$) was observed in experiment 2 when the probiotic bacteria were delivered through live food (Table 4), the increase ranging between 17% and 20% for BF2/10 and BF4/10 fish respectively. Survival was also higher ($P < 0.05$) at the highest

Table 3 Effects of administration mode and doses of *Bacillus* probiotics on growth and survival related parameters of Eurasian perch postlarvae reared under intensive conditions

	B0	BW	BF2W	BF4W
SGR (% day^{-1})				
d14	15.0 ± 0.9^{a}	15.9 ± 1.3^{a}	15.9 ± 0.1.1^{a}	17.9 ± 0.5^{b}
d28	16.5 ± 0.5^{a}	17.8 ± 0.5^{a}	18.3 ± 0.4^{a}	20.3 ± 0.5^{b}
FWH	1.27 ± 0.4^{b}	1.60 ± 0.2^{b}	0.63 ± 0.2^{a}	0.58 ± 0.2^{a}
Survival (%)	63.3 ± 13^{a}	55.7 ± 12^{a}	64.2 ± 2^{a}	67.0 ± 6^{a}
Mortality by Can II (%)	22.0 ± 16^{b}	29.0 ± 12^{b}	15.0 ± 2^{a}	14.4 ± 3^{a}

Values with different letters are significantly different at $P < 0.05$.
B0, control without probiotics; BW, water (4.93×10^{7} CFU L^{-1} day^{-1}); BWF2, water (4.93×10^{7} CFU L^{-1} day^{-1})+live food (2×10^{10} CFU L^{-1}); BWF4, water (4.93×10^{7} CFU L^{-1} day^{-1})+live food (4×10^{10} CFU L^{-1}); SGR, specific growth rate; FWH, final weight heterogeneity (= CV_{FBW}%/CV_{IBW}%); Can II, type II cannibalism (ingestion of whole prey); d14 and d28, days 28 and 42 post hatching.

Table 4 Effects of administration mode of *Bacillus* probiotics and stocking density level on growth and survival-related parameters of Eurasian perch postlarvae reared under intensive conditions

	B010	BF2/10	BF4/10	BW10	BW20	BF2W10	BF2W20
SGR (% day^{-1})							
d14	10.9 ± 0.7^{a}	11.1 ± 0.5^{a}	11.3 ± 0.4^{a}	11.0 ± 0.4^{a}	11.1 ± 0.3^{a}	11.5 ± 1.4^{a}	11.3 ± 0.2^{a}
d28	9.4 ± 0.5^{a}	10.2 ± 0.2^{a}	11.4 ± 0.2^{b}	9.4 ± 0.6^{a}	9.7 ± 0.4^{a}	9.5 ± 0.4^{a}	10.1 ± 0.2^{a}
FWH	0.28 ± 0.1^{b}	0.10 ± 0.0^{a}	0.09 ± 0.0^{a}	0.39 ± 0.1^{b}	0.25 ± 0.2^{b}	0.23 ± 0.2^{b}	0.15 ± 0.0^{a}
Survival (%)	35.3 ± 1.5^{a}	52.3 ± 5.5^{b}	54.7 ± 0.6^{b}	39.0 ± 8.5^{a}	53.3 ± 5.5^{b}	41.7 ± 12^{a}	52.7 ± 7.1^{b}
Mortality by Can II (%)	38.3 ± 7.6^{b}	19.7 ± 4.2^{a}	23.3 ± 4.0^{a}	30.0 ± 5.7^{ab}	28.0 ± 11^{ab}	42.3 ± 7.6^{b}	20.7 ± 7.1^{a}

Values with different letters are significantly different at $P < 0.05$.
B010, control without probiotics; BF2/10, live food (2×10^{10} CFU L^{-1}) (10 fish L^{-1}); BF4/10, live food (4×10^{10} CFU L^{-1}) (10 fish L^{-1}); BW10, water (4.93×10^{7} CFU L^{-1} day^{-1}) (10 fish L^{-1}); BW20, water (4.93×10^{7} CFU L^{-1} day^{-1}) (20 fish L^{-1}); BF2W10, live food (2×10^{10} CFU L^{-1})+water (4.93×10^{7} CFU L^{-1} day^{-1}) (10 fish L^{-1}); BF2W20, live food (2×10^{10} CFU L^{-1})+water (4.93×10^{7} CFU L^{-1} day^{-1}) (20 fish L^{-1}); SGR, specific growth rate; FWH, final weight heterogeneity (= CV_{FBW}%/CV_{IBW}%); Can II, type II cannibalism (ingestion of whole prey); d14 and d28, days 32 and 46 post hatching.

stocking density level (BW20 and BF2W20) whatever the administration mode, and this was associated by low type-2 cannibalism in BF2W20 fish but not in BW20 ones.

Immune variables

In both experiments, lysozyme activity (Fig. 1a and b) in the fish homogenates was not markedly affected by the probiotic bacteria whatever the administration mode or the dietary dose, except for a low increase (21%, $P < 0.05$) at the highest dietary dose on day 28 in experiment 1 (Fig. 1a). In contrast to lysozyme activity, a significant stimulation ($P < 0.05$) of total Ig production was observed in experiment 1 (Fig. 2a); when compared with B0, the significant increase varied between 72–111% and 61–65% in BFW fish and BW ones respectively. The same Ig response was observed in experiment 2 (Fig. 2b) with a dose-related response in BF fish. The highest Ig response was observed in the BF groups (115–191%) compared with BFW (64–103%) and BW (26–30%), indicating a low efficiency for bacteria administration through water. In both experiments, the Ig response was significantly higher ($P < 0.05$) on day 28 than on day 14, except for BW fish (Fig. 2a). The two immune compounds were not affected by stocking density whatever the administration mode in experiment 2.

Discussion

Husbandry variables

Results from the present study show that the mixture of *Bacillus* strains (SANOLIFE MIC-F®, INVE Aquaculture) used is a potential product in stimulating growth of Eurasian perch postlarvae depending on

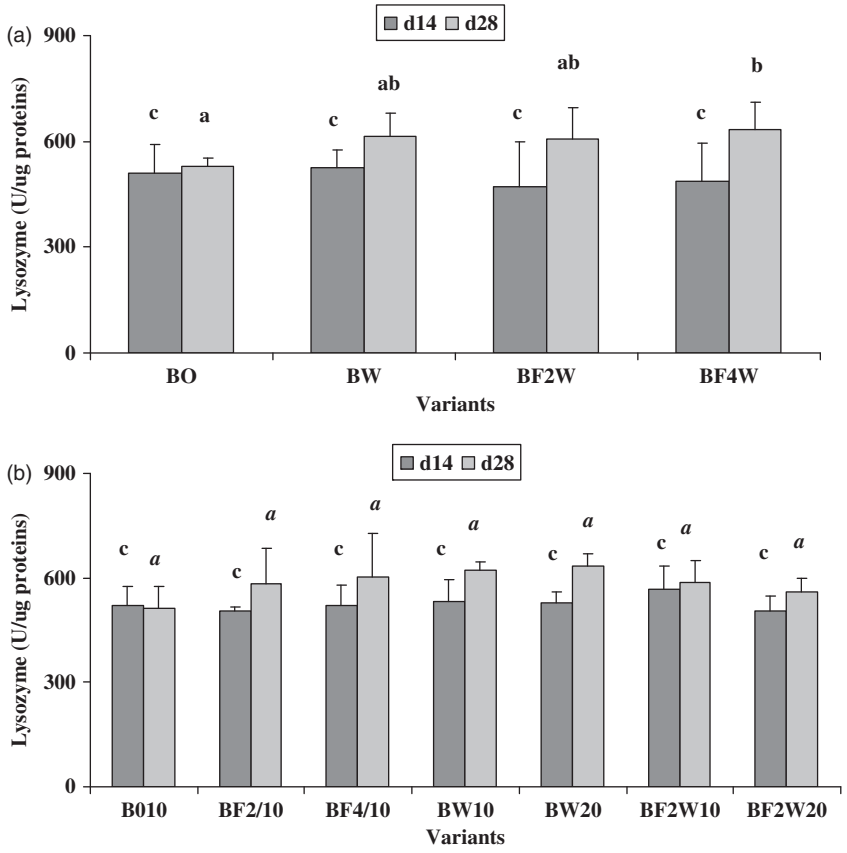


Figure 1 Effects of administration mode of *Bacillus* probiotics and stocking density level on lysozyme activity in the homogenates of Eurasian perch postlarvae. (a) and (b) Experiments 1 and 2. B0, control without probiotics; BW, water (4.93×10^7 CFU L^{-1} day^{-1}); BWF2, water (4.93×10^7 CFU L^{-1} day^{-1})+live food (2×10^{10} CFU L^{-1} day^{-1}); BWF4, water (4.93×10^7 CFU L^{-1} day^{-1})+live food (4×1010 CFU L^{-1} day^{-1}). Values with different letters are significantly different at $P < 0.05$.

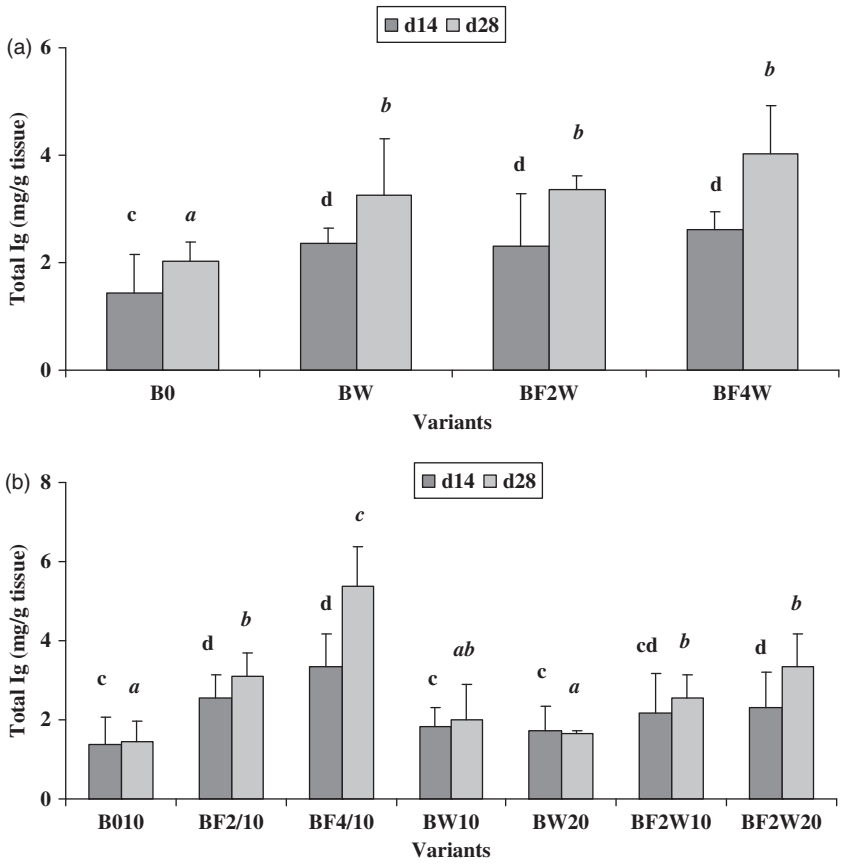


Figure 2 Effects of administration mode of *Bacillus* probiotics and stocking density level on total Ig level in the homogenates of Eurasian perch postlarvae. (a) and (b) Experiments 1 and 2. B0/10, control without probiotics; BF2/10, live food (2×10^{10} CFU L^{-1} day^{-1}) (10 fish L^{-1}); BF4/10, live food (4×10^{10} CFU L^{-1} day^{-1}) (10 fish L^{-1}); BW10, water (4.93×10^{7} CFU L^{-1} day^{-1}) (10 fish L^{-1}); BW20, water (4.93×10^{7} CFU L^{-1} day^{-1}) (20 fish L^{-1}); BF2W10, live food (2×10^{10} CFU L^{-1} day^{-1})+water (4.93×10^{7} CFU L^{-1} day^{-1}) (10 fish L^{-1}); BF2W20, live food (2×10^{10} CFU L^{-1} day^{-1})+water (4.93×10^{7} CFU L^{-1} day^{-1}) (20 fish L^{-1}).Values with different letters are significantly different at $P < 0.05$.

the dose and the administration mode. Indeed, the results from the two experiments showed a significant growth response when *Artemia* metanauplii were enriched with a dose of 4×10^{10} CFU L^{-1} incubation medium regardless of bacteria addition in the tank water. Enrichment of metanauplii with low bacteria dose or administration of bacteria through water only did not induce any significant growth increase. Investigations on the effects of probiotics in percid fish culture are scarce, but among several probiotic bacteria tested in fish, products containing *Bacillus* or *Lactobacillus* strains have been reported to stimulate growth in salmonid and cyprinid fish (see review by Kesarcodi-Watson *et al.* 2008; Apun-Molina *et al.* 2009). In addition, stimulating action of *B. pumilus* alone or a mixture of *Bacillus* strains added to a dry food has been recently reported in Nile tilapia fingerlings (Aly *et al.* 2008; Apun-Molina *et al.* 2009). Similar positive action with *L. acidophilus* was reported in African catfish *Clarias gariepinus* B. (Al-Dohail *et al.* 2009). The positive impact on growth by the same *Bacillus* mixture as used in the present study has been also reported in other fish species, namely gilthead seabream *Sparus aurata* L., Japanese flounder *Paralichthys olivaceus*, Senegalese sole and turbot *Psetta maximum* L. by Decamp, Makridis, Qi, Xin, Moriarty, Sorgeloos and Lavens (2006), Decamp, Yu, Xin and Morialty (2007), as well as in larvae of shrimp species, such as *Penaeus monodon* and *Litopenaeus vannamei* (Decamp *et al.* 2008). The mechanisms by which probiotic bacteria stimulate growth rate are not yet clearly elucidated. The available investigations indicate that some probiotics increase the content of digestive enzymes in the gastro-intest-

inal tract, and thereby facilitate nutrient utilization and digestion (Klemicki & Klewicka 2004; El-Haroun *et al.* 2006; Abdelhamid *et al.* 2009). Especially, the stimulating growth by probiotics containing *Bacillus* strains has been associated with improved feed conversion ratio and protein efficiency ratio attributed to an increase in lactic acid and cellulolytic and amylolytic enzyme production in rohu *L. rohita* (Bairagi, Sakar Ghosh & Sen Ray 2004; Kesarcodi-Watson *et al.* 2008). The nutrient absorption may also be improved because it has been reported that some *Bacillus* probiotics induce an increase in transferrin expression in the serum of rainbow trout *Oncorhynchus mykiss* W., as it is known that transferrin stimulates the iron absorption in the gut, and maintains the iron balance for cells in vertebrates (Yoshiga *et al.* 1997 cited by Brunt, Hansen, Jamieson & Austin 2008). In the present study, the feeding behaviour was standardized before the settings to provide the same amounts of live food to the fish during the bacteria administration. Thus, it is supposed that the higher stimulating growth action observed with the high dietary bacteria dose may result to a better food utilization or digestion, even if no parameters of these processes were measured in this study. Perhaps, the *in vivo* uptake of *Bacillus* bacteria by the fish in the case of administration through water was not enough to stimulate the digestive mechanisms as no significant growth response was observed. But a lack of efficiency for the *Bacillus* bacteria cannot be ruled out because competition with other types of bacteria may already occur in the recirculating water system, or to some extent, an intra-disruption in the *Bacillus* functionality in relation to the physicochemical conditions in the culture tanks.

As for growth, survival was affected by the way of bacteria administration. When administered through water, bacteria were not fully effective to improve fish survival, except for fish reared at higher density in experiment 2. In contrast, the dietary *Bacillus* probiotics seem to induce a decrease in mortality caused by type-2 cannibalism in relation to low-weight heterogeneity. Using the same probiotic bacteria as in the present study, Decamp *et al.* (2006) also demonstrated an increase in survival associated with low size variability. In fact, high size heterogeneity is an important issue affecting the emergence and intensity of type-2 cannibalism, which is one of the most important factors affecting survival in Eurasian perch postlarvae (Kestemont *et al.* 2003; Babiak *et al.* 2004; Mandiki *et al.* 2007). The reduction in size heterogeneity by the dietary probiotic bacteria may be an indirect effect related to the improvement of feed utilization or the increase in protein efficiency, which may also induce changes in cannibalistic behaviour. Concerning the effect of stocking density level on survival, previous studies reported that higher stocking densities inhibit cannibalism rates in larvae, thus resulting in higher survival; whereas in postlarvae, cannibalism rate is higher at high stocking densities with reduction in survival (Baras *et al.* 2003; Kestemont *et al.* 2003). In contrast to the latter reports, type-2 cannibalism was reduced with consequently high survival rate at the highest density level (20 fish L^{-1}) for postlarvae that received probiotic bacteria through live food only or through both live food and water (experiment 2). Such controversial results may be an indication for a positive interaction of the bacteria action with the stocking density by hampering the size heterogeneity-related cannibalism behaviour. However, besides cannibalism, other causes of mortalities should be taken into account because higher survival without a reduction in size heterogeneity was observed at the highest density in fish that received bacteria through tank water.

Immune status

Apart from the reduction in size heterogeneity, the increase in survival observed in the current study was associated with changes in specific immune defence, as evidenced by increased total Ig levels in both experiments, especially for bacteria administration through the live food or through live food associated with tank water. For the two experiments, the increase in total Ig levels was already detectable after 2 weeks whatever the bacteria dose for the dietary administration. The increase in total Ig levels was also observed for bacteria administration through tank water but only in experiment 1 not in experiment 2 for the two density levels tested (10 or 20 fish L^{-1}), indicating a low efficiency of bacteria delivered by tank water. An increase in survival by probiotic bacteria has been reported in some salmonid and cyprinid fish (Kesarcodi-Watson *et al.* 2008; Bandyopadhyay & Das Mohapatra 2009) but the related changes in immune defence have received little attention. It has been shown that probiotics containing *Lactobacillus* spp. increase survival as a result of improved immune defence in rainbow trout (Nikoskelainen *et al.* 2003; Panigrahia *et al.* 2005). Recently, changes in respiratory burst activity, Ig concentration, haematological parameters, and improved disease

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resistance were reported in Nile tilapia and African catfish fingerlings after using *B. pumilus* or *L. acidophilus* as probiotics (Aly *et al.* 2008; Al-Dohail *et al.* 2009). Probiotic bacteria may act on the immune defence by various ways, such as mitigating the immunosuppressive actions of some pathogens by production of antimicrobial substances (Yan, Boyd & Burges 2002; Ringo *et al.* 2010). Especially, it has been reported that some *Lactobacillus* or *Bacillus* spp. produce antimicrobial agents active against Gram-positive and -negative organisms (Rastall 2004; Wang *et al.* 2008). A competition by the probiotic bacteria for the attachment sites is also suggested as one of the action modes for enhancing immune defence and disease resistance even if little evidence has been reported in fish (Kesarcodi-Watson *et al.* 2008). Using the same probiotic bacteria as in the present study, Decamp *et al.* (2006) demonstrated an increase in survival associated with inhibition of pathogens such as *Vibrio* spp. in water and gastro-intestinal tract in gilthead seabream and Japanese flounder, but results concerning the mode of action are not yet published. Recently, Caipang, Brinchmann and Kiron (2010) demonstrated that some bacteria isolated from the gastro-intestinal tract of Atlantic cod had high *in vitro* antagonistic activity against *V. anguillarum* and *A. salmonicida* depending to the incubation temperatures. They also demonstrated discrepancies among the bacteria tested as probiotic candidates in triggering the expression of lysozyme gene in head kidney cells *in vitro*, indicating that there may be differences in the mechanisms of lysozyme expression due to the bacterial types. In rainbow trout, Panigrahia *et al.* (2005) did not observe significant differences in lysozyme activity between fish groups treated with different forms of *Bacillus rhamnosus*. Our results did not show a consistent effect on the non-specific immune defence because lysozyme activity was enhanced by high dietary bacteria dose in experiment 1 but this was not confirmed in experiment 2 by the same administration mode. Therefore, we can assume that the mixture of *Bacillus* species tested in the present study is not enough active to stimulate significantly lysozyme production during the post-larval stage in fish. Perhaps, higher doses than those tested are needed for a better efficiency regarding the non-specific immune system.

In conclusion, the results show that high dietary concentrations of the mixture of *Bacillus* species used have potential stimulating impact on growth, survival-related parameters and immune compounds, at least total Ig in Eurasian perch. But further investigations are needed to demonstrate the optimal doses for emphasizing immune defence status and disease resistance.

Acknowledgments

The authors are grateful to the INVE Aquaculture (Belgium) for providing the mixture of *Bacillus* SANOLIFE MIC-F®. They are also grateful to André Evrard, Marie-Claire Forget and Anne Vermeylen for their technical assistance. The research was partly funded by the Belgian National Funds for Science (FNRS, contract 2.4.568.00.F).

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